UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

TESIS DOCTORAL

Efecto de la introducción de genética Duroc y de la restricción de vitamina A en la dieta sobre parámetros productivos, expresión génica y calidad de la carne en el cerdo ibérico

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Miriam Ayuso Hernando

Directoras

Cristina Óvilo Martín
Beatriz Isabel Redondo
Ana Isabel Rey Muñoz

Madrid, 2016

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

UNIVERSIDAD
COMPLUTENSE
MADRID

EFECTO DE LA INTRODUCCIÓN DE GENÉTICA DUROC Y DE LA RESTRICCIÓN DE VITAMINA A EN LA DIETA SOBRE PARÁMETROS PRODUCTIVOS, EXPRESIÓN GÉNICA Y CALIDAD DE LA CARNE EN EL CERDO IBÉRICO

MIRIAM AYUSO HERNANDO

MADRID, 2015

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

EFECTO DE LA INTRODUCCIÓN DE GENÉTICA DUROC Y DE LA RESTRICCIÓN DE VITAMINA A EN LA DIETA SOBRE PARÁMETROS PRODUCTIVOS, EXPRESIÓN GÉNICA Y CALIDAD DE LA CARNE EN EL CERDO IBÉRICO

Tesis Doctoral presentada por
MIRIAM AYUSO HERNANDO

Realizada bajo la dirección de las doctoras
CRISTINA ÓVILO MARTÍN
BEATRIZ ISABEL REDONDO
ANA ISABEL REY MUÑOZ

MADRID, 2015

Las doctoras Ana Isabel Rey Muñoz y Beatriz Isabel Redondo, profesoras titulares del departamento de Producción Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid, y la doctora Cristina Óvilo Martín, investigadora del departamento de Mejora Genética Animal del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria,

CERTIFICAN:

Que la Tesis Doctoral titulada "EFECTO DE LA INTRODUCCIÓN DE GENÉTICA DUROC Y DE LA RESTRICCIÓN DE VITAMINA A EN LA DIETA SOBRE PARÁMETROS PRODUCTIVOS, EXPRESIÓN GÉNICA Y CALIDAD DE LA CARNE EN EL CERDO IBÉRICO" presentada por Miriam Ayuso Hernando para optar al grado de Doctor, ha sido realizada bajo su dirección, cumple las condiciones exigidas para obtener dicho título y autorizan su presentación para que sea juzgada por la comisión correspondiente.

Y para que así conste, firman en Madrid, a 14 de octubre de 2015.

Ana Isabel Rey Muñoz Beatriz Isabel Redondo Cristina Óvilo Martín

El trabajo experimental que ha dado lugar a esta memoria ha sido realizado en el Departamento de Producción Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid y en el Departamento de Mejora Genérica Animal del Instituto Nacional de Investigación Agraria y Alimentaria, financiado mediante los siguientes proyectos de investigación:

* AGL2010-21991-C03 del Ministerio de Ciencia e Innovación.

* Ayuda predoctoral BES-2011-045136 del Ministerio de Ciencia e Innovación.

* MEDGAN (S2013/ABI-2913), Comunidad de Madrid y CEI Campus Moncloa UCM-UPM.

* AGL2013-48121-C3 del Ministerio de economía y competitividad.

A mis directoras

A mi familia y amigos

"Nunca podrás escapar de tu corazón,
así que es mejor que escuches lo que tiene que decirte."

Paulo Coelho, "El alquimista"

AGRADECIMIENTOS

Quisiera expresar mi agradecimiento a las instituciones y a todas las personas que me han ayudado durante estos años en la realización de esta Tesis Doctoral.

Quiero agradecer en primer lugar a mis directoras de tesis, Cristina Óvilo Martín, Beatriz Isabel Redondo y Ana Isabel Rey Muñoz, la oportunidad de iniciar mi carrera como investigadora junto a ellas. También quiero agradeceros la enorme cantidad de tiempo, dedicación y esfuerzo que habéis dedicado a este trabajo y a mí. Gracias por vuestro apoyo, confianza y respeto, que han hecho que pueda desarrollarme en el ámbito profesional y personal, y por ser un referente en todo momento. Gracias por contagiarme vuestra pasión por este mundo.

También quiero agradecer al Dr. Clemente López Bote el haber sido mi tutor durante estos cuatro años, en los cuales me ha mostrado su visión de la ciencia. No siempre es fácil trabajar con una persona tan brillante, con miles de ideas, proyectos y con mucha gente alrededor, pero desde luego siempre es enriquecedor. Los estudiantes tenemos suerte de encontrarnos con un profesor en todo el sentido de la palabra, al que no sólo le encanta enseñar, sino también aprender. Por todo ello, y por haber vuelto de Estados Unidos, gracias.

Al Dr. Antonio González Bulnes, a quien admiro profundamente como investigador y como persona, gracias por tu apoyo en todos los momentos, sobre todo en los más duros de la tesis. Gracias por los momentos vividos, por tu humor y tu filosofía de vida. Por todos los conocimientos, por intentar que me *prenatal programe*, que mire más allá del jamón y sobre todo por la pasión con la que vives la investigación y que transmites a la gente que hay a tu alrededor.

A los doctores Luís Silió, Argimiro Daza, Ana Isabel Fernández y Almudena Fernández, porque sin su ayuda y apoyo este trabajo no hubiera sido posible.

A Isa, Raúl y Olga, compañeros del laboratorio de nutrición, sin los que este trabajo no hubiera sido posible, por su ayuda y por los buenos momentos.

A la gente del INIA (Susana, Mari Luz, Rita, Yolanda, Carmen, Estefania, Fabián, Ángel…), por su ayuda y por haber estado siempre dispuestos a enseñarme y ayudarme.

Gracias a los Doctores Mike Tokach, Juan F. Medrano y Alberto Conde Aguilera, por la oportunidad de realizar estancias en centros tan importantes como la universidad de Kansas, California y el INRA, por haber contribuido enormemente a que estas estancias se hayan convertido en una maravillosa experiencia de las más enriquecedoras que he tenido la fortuna de vivir. Gracias por haberme abierto las puertas de sus centros y de sus vidas, y por haberme hecho sentir como en casa.

A José, mi compañero de fatigas, por estar siempre dispuesto a arreglar el mundo, por las risas y las quejas y por ser mi profesor particular de química. De no ser por ti, aún no sabría ni encender el gases.

A la nueva hornada, Consuelo, Marta y Laura, por vuestra ilusión, los buenos momentos compartidos y por vuestros ánimos.

A mis amigas de Vallecas, por estar siempre ahí, por considerarme vuestra veterinaria por el mundo y por hacerme desconectar tras tan sólo cinco minutos a vuestro lado. A Silvia por leer esta tesis y por esforzarte en comprender qué es la adipogénesis.

A mis minivips, por haber entrado en mi vida como internos y haberos quedado como amigos de los de verdad. Gracias por los juernes, las pochas y los aquelarres. Gracias por vuestro humor, apoyo y por vuestros ánimos.

A mis padres tengo que agradecérselo todo. Gracias por el esfuerzo de toda una vida, que ha permitido que hoy pueda ser lo que soñaba de niña, por ser todo un ejemplo para mí y los mejores padres que puedo imaginar. A mi hermano, gracias por tu tiempo, porque cada rato que paso contigo es genial y también por ser un amigo al que conozco hace 25 años. A los tres, gracias por confiar siempre en mí y por animarme y, sobre todo, por ser el centro de mi vida.

Al resto de mi familia, que siempre me ha apoyado. En especial a mi tía Mari, por todo lo que haces siempre por nosotros.

A Ismael, por tu apoyo y tu comprensión infinita, por tu cariño, por tu sonrisa, por hacer que los peores momentos no parezcan tan horribles. Por confiar y creer en mí más que yo misma, por empujarme a ser mejor. Gracias por haber estado a mi lado a pesar de exámenes, guardias, congresos y estancias. Sabes que la mitad de mi carrera y de esta tesis son tuyas. Gracias por hacerme feliz.

INDICE

ÍNDICE DE FIGURAS

Figuras de la introducción

Figuras del capítulo 1

Figuras del capítulo 2

Figuras del capítulo 3

Figuras del capítulo 4

Figuras del capítulo 5

Figuras de la discusión general

ÍNDICE DE TABLAS

Tablas de la introducción

Tablas del capítulo 1

Tablas del capítulo 2

Tablas del capítulo 3

Tablas del capítulo 4

Tablas del capítulo 5

ABREVIATURAS UTILIZADAS

ACACA	*Acetyl-CoA carboxylase alpha (Acetil-CoA carboxilasa, alfa)*
ACOX1	*Acyl-CoA oxidase 1, palmitoyl (Acil-CoA oxidasa 1)*
ACSL4	*Acyl-CoA synthetase long-chain family member cuatro (Acil-CoA sintasa de ácidos grasos de cadena larga 4)*
ADH	*Alcohol dehydrogenase (Alcohol deshidrogenasa)*
AdPLA	*Adipose-specific phospholipase A2 (Fosfolipasa A2 especifica de tejido adiposo)*
AGMI	Ácidos grasos monoinsaturados
AGPI	Ácidos grasos poliinsaturados
AGRP	*Agouti-related protein (Proteína asociada a agutí)*
AGS	Ácidos grasos saturados
AR	Ácido retinoico
ASCs	*Adipose tissue-derived stromal cells* (Células estromales derivadas del tejido adiposo)
ASICI	Asociación Interprofesional de Cerdo Ibérico
ASXL2	*Additional sex combs-like protein*
BCMO1	*Beta-carotene 15,15-monooxygenase 1 (Beta caroteno monoxigenasa)*
BF	*Biceps femoris*
BMAL1/ ARNTL	*Aryl hydrocarbon receptor nuclear translocator-like (Proteína similar al translocador nuclear del receptor aril hidrocarburo*
CEBPA, β, δ	*CCAAT/enhancer binding protein, alpha, beta, delta (Proteínas de unión a CCAAT/enhancer)*
CART	*Cocaine and amphetamine regulated transcript (Transcrito regulado por anfetamina y cocaína)*
CHOP	*CCAAT-enhancer-binding protein homologous protein (Proteína homóloga a las proteínas de unión a CCAAT)*
CLA	*Conjugated linoleic acid* (Ácido linoleico conjugado)
CPT-1	*Carnitine-palmitoyl-transferase 1 (Carnitina pamitoil transferasa 1)*
CRBPII	*Retinyl ester hydrolase (Retinil éster hidrolasa)*
CREB	*CAMP responsive element binding protein (Proteína de unión al elemento de respuesta al CAMP)*
DE	Diferencialmente expresado
DGAT1	*Diacylglycerol O-acyltransferase 1 (Diacilglicerol aciltransferasa 1)*
DLK1	*Delta-like 1 homolog (drosophila) (Homólogo de la proteína similar a*

	delta)
ELOVL6	*Long-chain fatty-acyl elongase (Elongasa de ácidos grasos de cadena larga)*
EPA	*Eicosapentanoic acid* (Ácido eicosapentaenoico)
EPAS1	*Endothelial PAS domain protein 1 (Proteína del dominio endotelial PAS)*
ERK	*Extracellular signal-regulated kinase (Kinasa regulada por señales extracelulares)*
FABP4	*Fatty acid binding protein (Proteína de unión a ácidos grasos)*
FASN	*Fatty acid synthase (Sintasa de ácidos grasos)*
FEDNA	Fundación española para el desarrollo de la nutrición animal
FGF	*Fibroblast growth factor (Factor de crecimiento de fibroblastos)*
G3PDH	*Glycerol-3-phosphate dehydrogenase (Glicerol-3-fosfato deshidrogenasa)*
GATA	*Gata binding protein 1 (Globin transcription factor 1) (Factor de transcripción de globinas)*
GIM	Grasa intramuscular
GLUT4	*Glucose transporter (Transportador de glucosa)*
GWAS	*Genome wide association study (*Estudio de asociación del genoma*)*
IFNG	*Interferon, gamma*
IGF-1	*Insulin-like growth factor 1 (Factor de crecimiento similar a la insulina 1)*
IGF-2	*Insulin-like growth factor 2 (Factor de crecimiento similar a la insulina 2)*
IL-1	*Interleukin 1 (Interleucina 1)*
INS	*Insulin (Insulina)*
JAK	*Janus kinase*
KLF	*Kruppel-like factor (Factor similar a kruppel)*
LD	*Longissimus dorsi*
LEP	*Leptin (Leptina)*
LEPR	*Leptin receptor (Receptor de leptina)*
LRAT	*Lecithin Retinol Acyltransferase (Lecitina retinol aciltransferasa)*
LXRs	*Liver x receptor (Receptor hepático x)*
MAGRAMA	Ministerio de Agricultura, Alimentación y Medio Ambiente
MC4R	*Melanocortin cuatro receptor (Receptor de melanocortina 4)*
ME	*Malic enzyme (Enzima málico)*

MEK	*Mitogen-activated protein kinase kinase 1 (Kinasa de proteína kinasa activada por mitógenos*
mRNA	ARN mensajero
MTP	*Microsomal triglyceride transfer protein (Proteína de transferencia de triglicéridos microsomales)*
NPY	*Neuropetpide Y (Neuropéptido Y)*
NRC	*National Research Council*
PCA	*Principal components analysis (*Análisis de componentes principales*)*
PCK2	*Phosphoenolpyruvate carboxylase (Carboxikinasa de fosfoenolpiruvato)*
PDGF	*Platelet-derived growth factor beta polypeptide (Factor de crecimiento derivado de plaquetas)*
PGF	*Placental growth factor (Factor de crecimiento placentario)*
POMC	*Propiomelanocortin*
PPARG	*Peroxisome proliferator-activated receptor gamma (Receptores activados por proliferadores peroxisomales gama)*
PREF-1	*Preadipocyte factor -1 (Factor preadipocitario 1)*
PLRP2	*Pancreatic lipase-related protein 2 (Proteína asociada a la lipasa pancreática)*
PTL	*Pancreatic lipase (Lipasa pancreática)*
qPCR	*Quantitave PCR (Polymerase chain reaction) (*PCR (Reacción en cadena de la polimerasa) cuantitativa*)*
QTLs	*Quantitative trait locus (Locus* de caracteres cuantitativos*)*
RALDH	*Retinaldehyde-specific dehydrogenase type 2 (Retinaldehído deshidrogenasa)*
RAR	*Retinoic acid receptor (Receptor de ácido retinoico)*
REH	*Retinyl ester hydrolase (Hidrolasa de retinil éster)*
RNA-Seq	*RNA sequencing* (Secuenciación del ARN)
RXRA	*Retinoid x receptor (Receptor retinoide x)*
SCD1	*Stearoyl-CoA desaturase/delta-9-desaturase (Estearoil CoA desaturasa)*
SM	*Semimembranosus*
SNP	*(Single nucleotide polymorphism* (Polimorfismo simple)
SOX9	*Sry (Sex determining region y)-box 9*
SR-B1	*Scavenger receptor class b, type I (Receptor xxxxxx*
SREBP1C	*Sterol regulatory element binding protein 1c (Proteína de unión al*

	elemento regulador del esterol 1c)
STAT5	*Signal transducer and activator of transcription 5 (Transductor de señales y activados de la transcripción 5)*
STRA6	*Stimulated by retinoic acid 6 (Proteína estimulada por el ácido retinoico)*
SVC	*Stromal vascular fraction* (Estroma vascular)
T3	*Triiodothyronine* (triyodotironina, hormona tiroidea)
TACE	*Tumor necrosis factor, alpha, converting enzyme (enzima convertidora del factor de necrosis tumoral alfa)*
TGF	*Transforming growth factor (factor de crecimiento transformante)*
TNFα	*Tumor necrosis factor (factor de necrosis tumoral)*
TPA	*Tissue plasminogen activator (activador de plasminógenos del tejido)*
TRα1	*Transformation/transcription domain-associated protein (Proteína asociada a dominios de transformación y trascripción)*
UI/IU	Unidades internacionales/International units
UPS	*Ubiquitin proteasome system* (Sistema ubiquitina-proteasoma)
VA	Vitamina A

RESUMEN/ABSTRACT

RESUMEN

El cerdo ibérico es una raza porcina que se caracteriza por una alta tasa de acumulación de grasa y por una capacidad de crecimiento magro limitada. Su carne tiene un alto contenido en grasa y un perfil de ácidos grasos característico que determina la alta calidad de sus productos. Esta raza ha sido cruzada con machos Duroc de forma habitual para mejorar el rendimiento productivo y el desarrollo muscular. Esta práctica disminuye la grasa intramuscular (GIM) y el contenido en ácidos grasos monoinsaturados (AGMI), parámetros asociados con la calidad de la carne. Estudios previos han demostrado que la genética y la nutrición entre otros, son factores clave que determinan la cantidad y composición de la GIM.

El objetivo general de la presente Tesis Doctoral ha sido profundizar en el conocimiento de los mecanismos genéticos y moleculares asociados a caracteres fenotípicos de interés en el cerdo ibérico, especialmente el contenido y composición de la GIM, así como conocer la respuesta de estos mecanismos ante estrategias nutricionales, concretamente la restricción de vitamina A.

Para la consecución de estos objetivos, se diseñaron dos ensayos experimentales. En el primero, se estudió el efecto del tipo genético y de la edad sobre el fenotipo y el transcriptoma muscular de cerdos ibéricos puros y cruzados con Duroc. Las etapas de desarrollo empleadas fueron el nacimiento y los cuatro meses de edad. Se analizaron diversos caracteres fenotípicos de interés y el transcriptoma de los músculos *Longissimus dorsi* (LD, en ambas edades) y *Biceps femoris* (BF, al nacimiento).

En el segundo ensayo, se estudió el efecto de la suplementación (10,000 UI/kg pienso) y la restricción (0 UI/kg pienso) de vitamina A (VA) sobre caracteres de interés, y sobre la expresión de genes candidato. La restricción de VA comenzó a los 2 (restricción temprana) o cuatro meses de edad (restricción tardía) y sus efectos se estudiaron a lo largo del crecimiento y cebo.

Los resultados del primer ensayo revelaron diferencias fenotípicas importantes entre tipos genéticos desde el nacimiento. Los cerdos puros fueron más pequeños en esta etapa y presentaron mayores niveles de colesterol que los cruzados. Sin embargo, estas diferencias no se observaron a los cuatro meses de edad. Por otro lado, los neonatos puros mostraron una mayor cantidad de GIM que los cruzados en el músculo BF al nacimiento y en el LD a los cuatro meses de edad. La composición de la GIM fue muy similar en ambos tipos genéticos. En cuanto al análisis del transcriptoma, la edad fue el factor que más afectó a la expresión génica, con 5,812 genes diferencialmente expresados (DE). El tipo genético y el músculo tuvieron un efecto menor (entre 113 y 261 genes DE). Se identificaron numerosos genes relacionados con el metabolismo lipídico que se encontraban afectados por la edad y el tipo genético (*DLK1, FGF21* o *NFAT*). El análisis

funcional reveló el músculo de los animales al nacimiento muestra un enriquecimiento en rutas y funciones relacionadas con procesos anabólicos y con el desarrollo que no se observa a los cuatro meses de edad. En ambos músculos, los cerdos puros mostraron una mayor activación de rutas relacionadas con el metabolismo lipídico y con el catabolismo de proteínas, especialmente al nacimiento. Sin embargo, los cerdos puros de cuatro meses de edad mostraron enriquecimiento de rutas y funciones asociadas al crecimiento muscular, similares a las observadas en neonatos cruzados. Este ensayo ha permitido también identificar numerosos genes reguladores (*EGRs, PPARGC1B, FOXOs, TRIM63, MYOD1* o *MEFs*) que podrían jugar un papel fundamental en el desarrollo de las diferencias fenotípicas observadas. Además, el estudio de polimorfismos realizado en estos reguladores identificó variantes no sinónimas en algunos de ellos, por ejemplo *PPARGC1B* y *TRIM63.*

En conclusión, los resultados obtenidos en este ensayo mejoran la comprensión de las diferencias metabólicas entre cerdos ibéricos puros y cruzados y resaltan el potencial de las técnicas de secuenciación masiva del ARN para identificar genes, rutas metabólicas y factores de transcripción involucrados en la variabilidad fenotípica para caracteres de interés económico.

El segundo ensayo evidenció un claro efecto de la restricción de VA sobre su acumulación tisular, aumentando en los animales suplementados, mientras que los cerdos restringidos mostraron una depleción de las reservas. Se observó una correlación negativa entre los niveles de vitaminas A y E, cuya acumulación pareció verse más afectada durante el periodo de crecimiento (alrededor de los ocho meses de edad). Esta observación coincidió con la expresión diferente de genes relacionados con el metabolismo de las vitaminas A y E. No se observaron diferencias en los parámetros productivos globales de los animales restringidos y suplementados al final del ciclo (11 meses de edad). A los cuatro meses, los animales restringidos mostraron un mayor número de preadipocitos que se tradujo en un mayor contenido en GIM en los músculos *semimembranosus* y LD de animales restringidos a los 11 meses de edad. La restricción de VA también aumentó la cantidad de AGMI y disminuyó la de ácidos grasos saturados en todos los tejidos muestreados excepto en el hígado. Estos efectos son más marcados cuando la VA se retira de la dieta a los dos meses de edad. La VA modificó la expresión de genes candidato (*ACSL4*, *CEBPB*, *IGF1*, *CRABPII* o *SCD*) relacionados con el metabolismo de la VA, la adipogénesis y el metabolismo lipídico.

Los resultados presentados en esta Tesis Doctoral aportan información novedosa de utilidad en el ámbito de la investigación genética básica, así como de la nutrición porcina.

ABSTRACT

The Iberian pig is a swine breed characterized by high fat deposition rate and limited capacity for lean tissue accretion. Its meat has a high fat content and a characteristic fatty acid profile, main factors determining the high quality of Iberian pig products. In order to improve productive performance and muscle development, this breed has been traditionally crossed with the Duroc breed. However, a decrease in intramuscular fat (IMF) and monounsaturated fatty acids (MUFA) content, parameters closely linked to meat quality, has been reported in those crossbred pigs. Previous studies showed than genetics and nutrition among others, are key factors controlling IMF content and composition.

Hence, the main goal of this Thesis was to contribute to the knowledge on genetic and molecular mechanisms associated with target phenotypic traits in Iberian pig, specially the intramuscular fat content and composition, and to study the effect of a nutritional strategy, specifically dietary vitamin A restriction, on these mechanisms.

Two different experiments were designed to achieve these objectives. In the first one, the effects of age and genotype on muscle transcriptome were studied in pure and Duroc-crossbred Iberian pigs at two developmental stages (birth and four months of age). Several phenotypic parameters and *Longissimus dorsi* (LD, at both ages) and *Biceps femoris* (BF, at birth) transcriptome were assessed.

The second experiment allowed the study of vitamin A supplementation (10,000 IU/kg feed) and restriction (0 IU/kg feed) on target traits and gene expression. Experimental diets administration started at two (early restriction group) or four (late restriction group) months of age and their effects were studied through the growing and fattening phases.

Results of the first experiment revealed significant phenotypic differences between genetic types from birth. Purebred Iberian showed smaller size and higher cholesterol plasma levels at this developmental stage than crossbred pigs. However, these differences were not evident at four months of age. Intramuscular fat content was higher in purebred newborns and in four months old purebreds in BF and LD muscles, respectively, but IMF composition was similar between genotypes. Regarding muscle transcriptome, age had the biggest effect on gene expression, since 5,812 genes changed their expression levels over time. Muscle and genetic type showed a slighter effect on gene expression (affected genes ranged from 113 to 261). Several genes involved in lipid metabolism (*DLK1, FGF21* or *NFAT*) were affected by age and genetic type. The biological interpretation revealed that active pathways in newborn but not in four months old pigs were associated with anabolic and developmental processes. Moreover, purebred pigs showed enrichment of pathways related to lipid metabolism and protein catabolism in both muscles, which was more marked at birth. At four months of age, activated pathways in those pigs were involved

in muscle growth similarly to results observed in crossbred newborns. Several regulators (*EGRs, PPARGC1B, FOXOs, TRIM63, MYOD1* or *MEFs*) that may play a role in the development of the observed phenotypic differences were identified. Additionally, a variant calling analysis was performed on significant regulators and disclosed several non-synonymous variants in transcription factors as *PPARGC1B* and *TRIM63*.

In conclusion, the results obtained in this experiment improve the understanding of metabolic differences between purebred and crossbreed Iberian pigs and highlight the potential of RNA-Seq technology to id
entify genes, metabolic pathways and transcription factors involved in phenotypic variability of economically relevant traits.

The second experiment demonstrated a noteworthy effect of vitamin A restriction on tissue vitamin A accumulation, which increased with supplementation time, while restricted animals suffered depots depletion. Also, a negative correlation between vitamins A and E storage was observed. Vitamins A and E accumulation seemed to be more responsive to dietary changes at eight months of age, in agreement with the differential expression of genes involved in their metabolism. No effect of vitamin A level was observed on productive performance at the end of the production cycle. However, four months old restricted pigs showed an increase in preadipocyte number, which agrees with the higher IMF content in LD and semimembranosus muscles in these pigs at the end of the fattening phase (11 months of age). Dietary vitamin A restriction increased MUFA and decreased saturated fatty acids concentration of all studied tissues, except the liver, in 11 months old restricted pigs. These effects are more marked when dietary treatment starts at two rather than four months of age. Also, vitamin A inclusion level modified expression of several candidate genes (*ACSL4, CEBPB, IGF1, CRABPII* or *SCD*) related to vitamin A and lipid metabolism and adipogenesis.

The results presented in this Thesis provide new and useful insights in basic genetics research and swine nutrition fields.

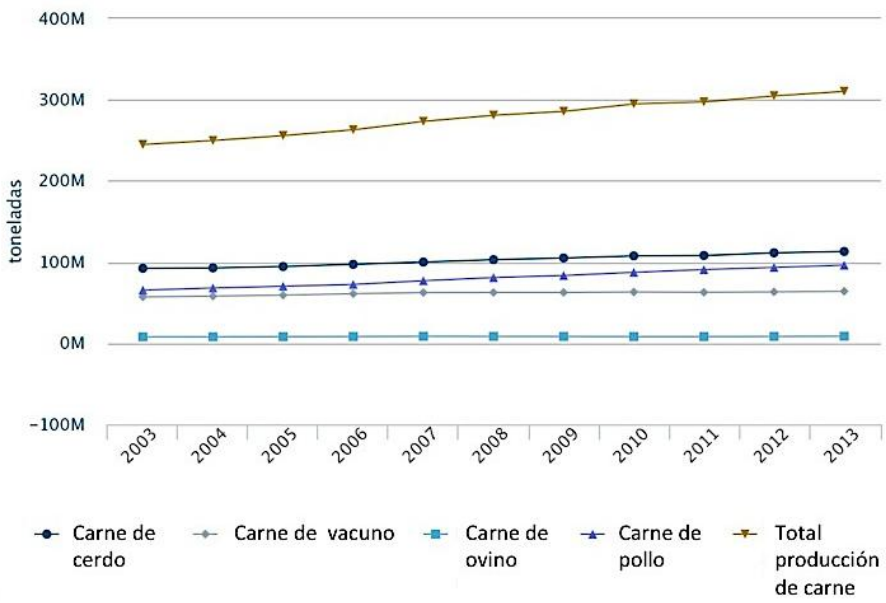
1.- INTRODUCCIÓN

1.1- SITUACIÓN ACTUAL DE LA PRODUCCIÓN MUNDIAL DE CARNE:

La carne es el producto pecuario de mayor valor. Posee proteínas y aminoácidos, minerales, grasas, vitaminas y otros componentes bioactivos, que le confieren su importancia nutricional, así como pequeñas cantidades de carbohidratos. La producción de carne consiste en la transformación de alimentos de origen vegetal, con elevado contenido en carbohidratos y proteínas, en tejidos animales formados casi exclusivamente por proteínas de alta calidad y por grasa.

Según datos recogidos en el informe de la subdirección general de productos ganaderos del Ministerio de Agricultura, Alimentación y Medio Ambiente (MAGRAMA), la carne de cerdo es la más producida en el ámbito mundial. Los 110,346 miles de toneladas de carne de cerdo producidos representan en torno al 40% del total en el año 2014, como se observa en la figura 1.

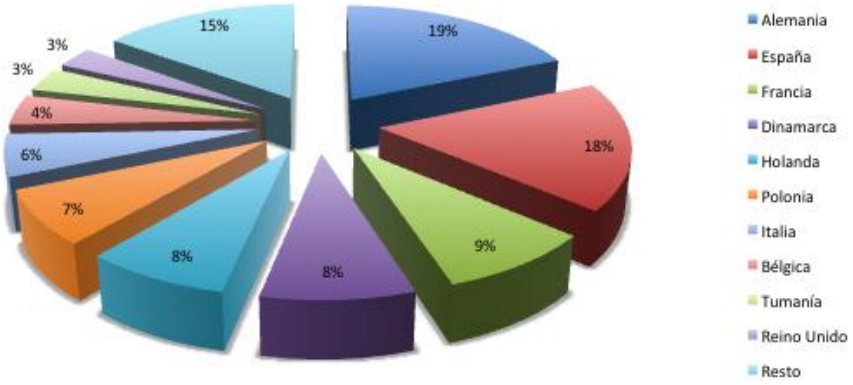
Figura 1: Evolución de la producción de carne en el ámbito mundial durante el periodo 2003-2013. (Fuente: FAOSTAT)



El continente asiático ha sido en 2014, como en años anteriores, el líder en cuanto a producción de carne de porcino. El continente europeo, con una producción de 22,250 miles de toneladas el año 2014, continúa siendo el segundo productor. China es el primer país productor, seguido de Estados Unidos.

Dentro de la Unión Europea-28, durante el año 2014, el censo de ganado porcino ha sido de 148,309 miles de cabezas. Alemania y España son los países con un mayor censo de porcino, como se observa en la figura 2.

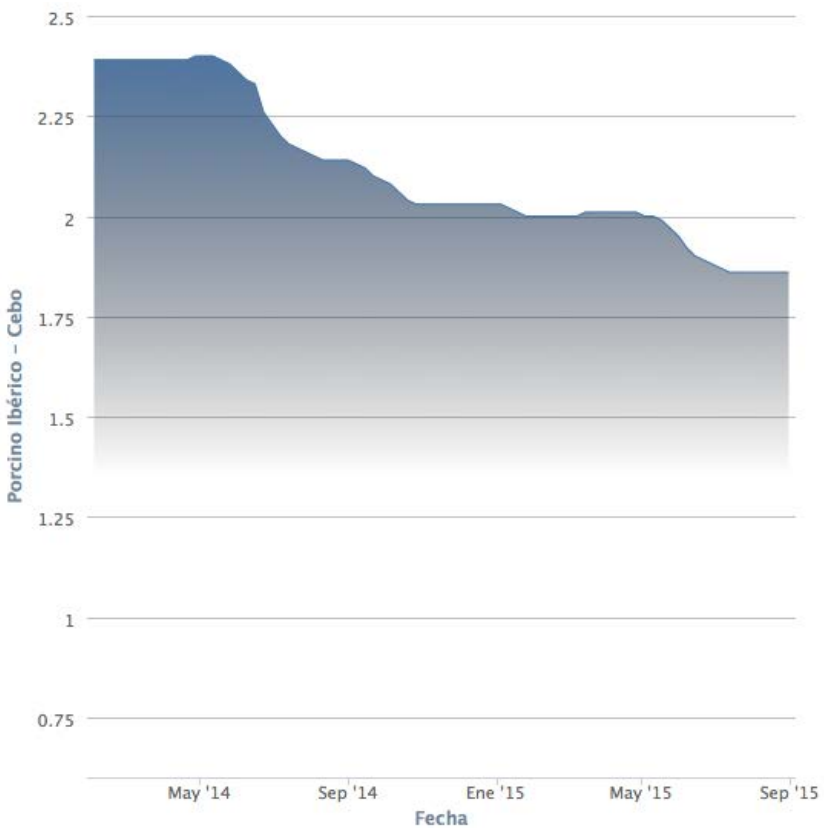
Figura 2: Censo de ganado porcino en la Unión Europea-28 en el año 2014. Representación porcentual de los principales países productores. (Fuente: EUROSTAT, 2015)



1.2- SITUACIÓN ACTUAL DE LA PRODUCCIÓN DE CARNE DE CERDO EN ESPAÑA:

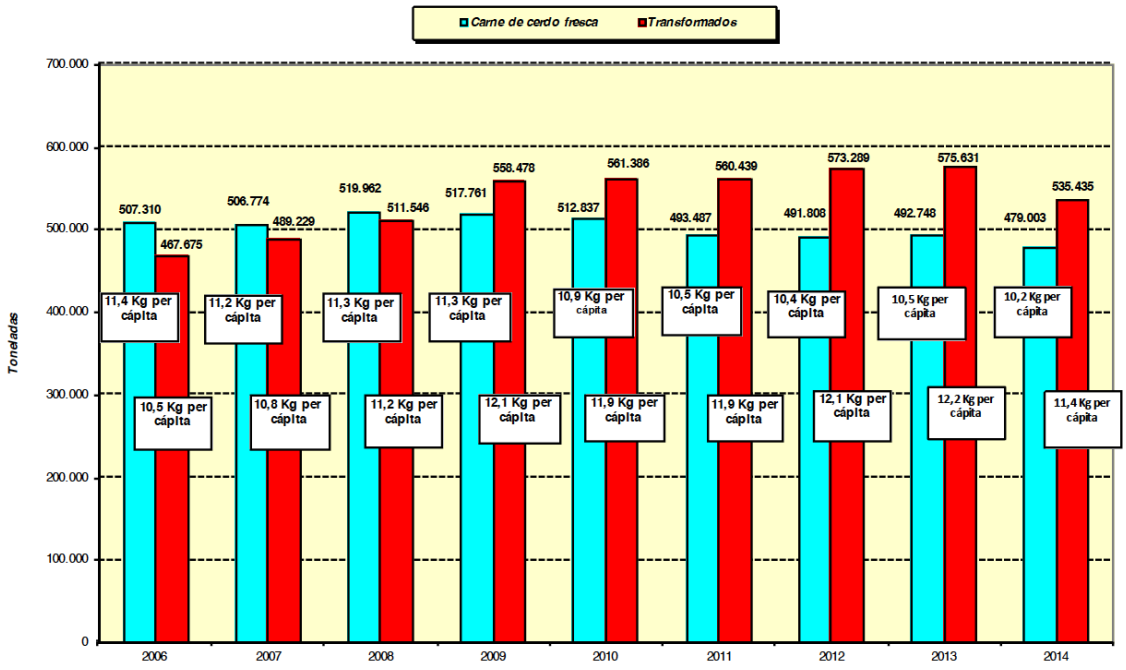
Según datos censales del Ministerio de Agricultura, Alimentación y Medio Ambiente de 2014, España cuenta con más de 26.5 millones de cabezas de porcino, de los cuales 2,175 miles cabezas corresponden al cerdo ibérico. Durante el año 2014, en España se han sacrificado aproximadamente 43.2 millones de cabezas de porcino, los cuales han aportado 3,571 miles de toneladas de carne. Los costes de producción de la carne de cerdo blanco se encuentran entre 1.08 y 1.10 euros/kg peso ganado, mientras que en cerdo ibérico, estos costes aumentarían hasta 1.39 euros/kg de peso repuesto en condiciones intensivas, tal y como se estimó en el año 2011 (http://feagas.com/images/stories/portal/actividades/cursos/2011/ponencias%20porcinas/07_JavierLlamazares.pdf). El estrecho margen de beneficios se traduce en unos precios de venta en torno a 1.14 euros/kg cerdo vivo en el caso del cerdo blanco, mientras que en porcino ibérico se sitúa en torno a 1.86 euros/kg, tras sufrir un descenso durante el periodo mayo 2014 – septiembre 2015 (Figura 3).

Figura 3: Evolución de la carne de porcino ibérico durante los años 2014-2015 (Fuente: lonja de Salamanca).



El precio de la carne de cerdo la hace relativamente accesible a toda la población (el precio de la carne de vacuno se sitúa en torno a los 4 euros/kg según la lonja de Salamanca) y favorece su consumo. De hecho, es la carne más consumida en los hogares españoles. Durante el año 2014, los españoles han consumido una media de 10.2 kg / cápita de carne de cerdo fresca, aumentando el consumo hasta 11.4 kg /cápita en el caso de los productos cárnicos (Figura 4), de modo que el total de carne de cerdo consumida es de 21.6 kg / cápita al año, mientras que en el caso de la carne de ave el consumo ha sido de 14.6 kg / cápita. Con un consumo bastante inferior se encuentra la carne de vacuno (6.6 kg / cápita) y la de ovino / caprino (2.1 kg / cápita). Finalmente, la carne de conejo es la menos consumida, con una media para el año 2011 de 1.34 kg / cápita (Informe anual del Observatorio de Consumo y de la Distribución Alimentaria, MAGRAMA).

Figura 4: Evolución del consumo de carne de cerdo fresca (barras azules) y derivados (barras rojas) por habitante en España. (Fuente: MAGRAMA)



Además de la importancia que tiene la carne de cerdo dentro de los hogares españoles, de los 3,751 miles de toneladas de carne producidas, en torno a un 42% (1,500 miles de toneladas) son exportadas cada año. Los principales destinatarios de las exportaciones españolas de carne de cerdo son países pertenecientes a la Unión Europea, mayoritariamente Francia, Portugal e Italia. Fuera de la Unión Europea, China es el principal receptor de carne de porcino de origen español. Estos datos reflejan claramente la importancia de la producción de carne de cerdo en España.

1.3- EL CERDO IBÉRICO

El sector del porcino ibérico tiene una gran importancia económica dentro de la producción ganadera española, especialmente en la zona adehesada. El censo más reciente (Mayo 2014) establece en 2,175,022 el número de cabezas de porcino ibérico, distribuidas por comunidades autónomas como se muestra en la Tabla 1.

Tabla 1: Censo de ganado porcino ibérico en España por comunidades autónomas en los años 2013 y 2014. (Fuente: MAGRAMA, 2014)

	2013	2014
MADRID	297	268
CASTILLA Y LEÓN	538,215	655,16
CASTILLA-LA MANCHA	57,858	57,562
EXTREMADURA	762,491	919,246
ANDALUCÍA	448,764	542,785
ESPAÑA	1,807,625	2,175,022

Pese a haber experimentado un descenso gradual desde el año 2008, el censo de porcino ibérico podría estar experimentando una recuperación. El número de cabezas ha aumentado entre los años 2013 y 2014, como se observa en la tabla 1. Extremadura se sitúa a la cabeza como primera comunidad de cría de cerdo ibérico, seguida por Castilla y León y Andalucía.

1.3.1- Características de la raza:

El cerdo ibérico deriva del *sus mediterraneous* (Aparicio Sánchez, 1960; Dieguez, 1992), y se caracteriza por presentar una gran variabilidad, debido a su escasa o nula selección. Las variedades negra y retinta son las más abundantes, pero también podemos encontrar variedades rubias o manchadas, con o sin pelo (lampiño). A pesar de esta variabilidad, en general se puede decir que el cerdo ibérico es un cerdo de conformación redondeada, con gran capacidad adipogénica, crecimiento lento y gran rusticidad, lo que hace posible su adaptación al medio físico de la dehesa y el aprovechamiento de sus recursos naturales.

Al tratarse de una raza poco seleccionada, sus parámetros productivos, reproductivos y de características de la canal, son peores que las obtenidas en razas modernas y más seleccionadas (López-Bote, 1998).

El sistema de producción tradicional se basa en el sacrificio a pesos elevados (mínimo 108 y 115 kg para ibéricos puros y cruzados, respectivamente) y con una edad mínima de 10 a 14 meses, debido a que son destinados principalmente a la elaboración de productos curados. La consistencia de la carne aumenta con la edad por un sobrecruzamiento progresivo del colágeno muscular (Mayoral et

al., 1999). Probablemente este factor (sin duda también influido por el ejercicio) sea importante en el mantenimiento de la textura de los productos cárnicos del ibérico, especialmente teniendo en cuenta que la grasa del cerdo ibérico presenta un alto grado de insaturación, lo que disminuye su consistencia. Por otra parte, el contenido en grasa, incluyendo la de infiltración, aumenta tanto con el peso como con la edad de sacrificio, mejorando de esta forma las características organolépticas del producto elaborado.

El cerdo ibérico se caracteriza además por una acumulación de proteína comparativamente menor y una acumulación de grasa superior a las observadas en otras razas porcinas, seleccionadas para alcanzar una eficiencia elevada en el crecimiento y en la acumulación de proteína (Barea et al., 2007). Por otro lado, el cerdo ibérico presenta niveles plasmáticos de leptina superiores a los encontrados en cerdos magros (Fernandez-Figares et al., 2007). Asimismo, se ha descrito una variante estructural en el gen del receptor de leptina (*LEPR*), con un alelo (*LEPR c.1987T*) fijado en la raza ibérica que tiene efectos sobre el apetito, crecimiento tardío y engrasamiento (Ovilo et al., 2005) y que influye en la regulación a nivel hipotalámico del balance energético (Óvilo et al., 2010). Los altos niveles plasmáticos de leptina, junto con el mayor apetito y engrasamiento observados en esta raza, son características relacionadas en medicina humana con el síndrome de resistencia a la leptina (Myers et al., 2008).

Estas particularidades en su metabolismo favorecen una mayor acumulación de tejido adiposo subcutáneo y una mayor infiltración de grasa en las masas musculares. Por ejemplo, en una comparación entre cerdo ibérico y Landrace, el espesor del tocino dorsal fue de 48.1 mm en ibéricos frente a 20.7 mm en los cerdos Landrace (Serra et al., 1998). En el mismo estudio, los cerdos ibéricos mostraron un contenido en grasa intramuscular (GIM) medio de 3.91%, mientras que dicho valor fue de 0.66% en los cerdos Landrace. Además del mayor contenido graso, el cerdo ibérico presenta un perfil de ácidos grasos característico, con mayor concentración de AGMI, principalmente ácido oleico que los observados en Landrace x Large White (45.6% de ácido oleico en ibérico frente a 44% en el cruce magro) (Barea et al., 2013) y en Landrace (48.9% de ácido oleico en ibérico frente a 46.5% en Landrace (Serra et al., 1998). Estas características proporcionan a la carne una textura, aroma y sabor únicos y excepcionales.

1.3.2- Productos cárnicos elaborados:

Las principales razones de la alta calidad de los productos elaborados procedentes del cerdo ibérico son la gran calidad de la materia prima y el cuidado puesto en el proceso de curado y de conservación, que supone una fase de salado, seguida de postsalado (durante el cual tiene lugar la redistribución de la sal por todo el producto, disminuyendo así la actividad de agua en el mismo) y a continuación, el secado de las piezas. Una vez secos, los productos permanecen en bodegas, donde

terminan de adquirir sus características organolépticas (Ventanas y Andrés, 2001). La duración de este proceso puede variar, pero el RD4/2014 exige un mínimo de 600 días de curación para jamones de menos de 7 kg de peso y de 730 días para jamones más pesados. Esto favorece el desarrollo del aroma, sabor y otras características óptimas del producto final.

Como se ha mencionado antes, las características de la materia prima, es decir, la carne de cerdo, también contribuyen a la calidad del producto final. El contenido en GIM, así como su composición (en concreto la cantidad de ácido oleico), son factores determinantes en la calidad de los productos ibéricos (Ventanas et al., 2005).

Los principales productos curados que se obtienen del cerdo ibérico son: jamón, paleta y embutidos curados.

Además, los productos procedentes de animales ibéricos se enmarcan dentro de la norma de calidad para la carne, el jamón, la paleta y la caña de lomo ibérico (Real Decreto 4/2014), que establece las siguientes denominaciones:

a) Designación por alimentación y manejo:

«De bellota»: Engloba todos los productos procedentes de animales sacrificados inmediatamente después del aprovechamiento exclusivo de bellota, hierba y otros recursos naturales de la dehesa, sin aporte de pienso suplementario y bajo condiciones de manejo específicas.

«De cebo de campo»: Engloba los productos procedentes de animales que aunque hayan podido aprovechar recursos de la dehesa o del campo, han sido alimentados con piensos constituidos fundamentalmente por cereales y leguminosas, y cuyo manejo se realice en explotaciones extensivas o intensivas al aire libre, pudiendo tener parte de la superficie cubierta.

«De cebo»: Engloba los productos procedentes de animales alimentados con piensos, constituidos fundamentalmente por cereales y leguminosas, cuyo manejo se realice en sistemas de explotación intensiva

b) Designación por tipo racial:

«100% ibérico»: Cuando se trate de productos procedentes de animales con un 100% de pureza genética de la raza ibérica, cuyos progenitores tengan así mismo un 100% de pureza racial ibérica y estén inscritos en el correspondiente libro genealógico.

«Ibérico»: Cuando se trate de productos procedentes de animales con al menos un 50% de su genética correspondiente a la raza porcina ibérica, siendo siempre la madre 100% ibérica y estando inscrita en el correspondiente libro genealógico.

Como se muestra en la Tabla 2, el grueso de la producción de cerdo ibérico en España corresponde a animales cruzados ibérico x Duroc, que suponen cerca del 93% de la producción total y a animales alimentados con pienso, bien en condiciones extensivas o intensivas.

Tabla 2: Animales comercializados bajo la norma de productos ibéricos en el año 2014 en las distintas comunidades autónomas. (Fuente: Informe anual ASICI, 2014)

	IBP llota	IBP mpo	IBP bo	IB llota	IB mpo	IB cebo	Total
Andalucía	71.694	5.894	3.531	79.046	45.533	114.572	320.270
C-La Mancha	2.455	124	3.559	2.100	2.567	208.867	219.672
C-León	2.513	13.241	3.182	37.560	58.349	798.609	913.454
Cataluña	0	0	582	0	0	63.494	64.076
Extremadura	44.926	2.689	4.136	177.969	185.697	347.017	762.434
Madrid	37	0	0	0	0	0	37
Murcia	0	0	0	0	0	101.137	101.137
TOTAL	121.625	21.948	14.990	296.675	292.146	1.633.696	2.381.080
	Total ibérico puro 158.563 Total ibérico 2.222.517						
	Total bellota: 418.300; Total cebo de campo: 314.094;						
	Total cebo: 1.648.686						

1.4- LA GRASA INTRAMUSCULAR:

1.4.1- Importancia

Entre los depósitos grasos animales, podemos diferenciar tres grandes tipos: grasa subcutánea o de cobertura (60-70% del total), la grasa visceral (10-15%) y la grasa inter e intramuscular (20-35%), que es aquella que se deposita tanto entre las fibras musculares como dentro de ellas, en forma de membranas lipídicas y pequeñas vacuolas grasas (Gerbens, 2004). La GIM o de veteado se ha relacionado con la calidad de la carne, proporcionando aroma, sabor y jugosidad.

En bibliografía anglosajona y centroeuropea se encuentran recomendaciones que indican que para percibir cambios en las cualidades sensoriales, es preciso que la carne contenga al menos un 2% de GIM (Barton-Gade y Bejerholm, 1985; Fernandez et al., 1999). Sin embargo, en España las costumbres gastronómicas y la tradición en el procesado de la carne hacen que se prefieran carnes con un contenido superior. En un estudio realizado por Cilla y colaboradores (Cilla et al., 2006) en el que se evaluó la calidad de jamones curados (DO Teruel) mediante un panel de cata profesional, los jamones con un contenido mayor de GIM en el músculo Semimembranoso (GIM = 4.7%) recibieron una puntuación mayor que jamones con un contenido en GIM menor (GIM = 4.4% y 4.1%). De este resultado se desprende que en España el contenido en GIM, al menos en productos curados, debe ser superior a un 4.4% para lograr la máxima aceptabilidad por parte de los consumidores.

Actualmente, el sector porcino está interesado en aumentar la cantidad y modificar la composición de la GIM. Esto es debido a que la cantidad y composición de la GIM juega un papel importante tanto en la calidad organoléptica (DeVol et al., 1988; Ellis et al., 1996; Fernandez et al., 1999) como en la salud humana, ya que ciertos ácidos grasos son beneficiosos desde el punto de vista de la salud cardiovascular (Hartog et al., 1987; Schmidt y Dyerberg, 1994).

1.4.2- Adipogénesis

La adipogénesis es el proceso de diferenciación de células grasas precursoras (preadipocitos) hacia células grasas maduras (adipocitos). Los adipocitos son las células mayoritarias en el tejido adiposo y se clasifican en tres tipos:

Adipocitos pardos: Se especializan en la producción de calor a partir de su almacenamiento lipídico y se encuentran únicamente en mamíferos. Difieren de los adipocitos blancos en la expresión de la proteína desacoplante 1 (UCP-1). Morfológicamente, los adipocitos pardos son multiloculares y contienen menos lípidos que los blancos, siendo particularmente ricos en mitocondrias. Este tipo de adipocitos son abundantes en recién nacidos y su número tiende a desaparecer con el tiempo.

Adipocitos blancos: son los adipocitos más abundantes. Son células esféricas que adquieren una característica forma de "anillo" cuando se encuentran llenos de lípidos, debido a que éstos desplazan el núcleo celular hacia la periferia. Constituyen el tejido adiposo blanco, el mayor reservorio energético en los mamíferos. Entre sus funciones principales destacan la síntesis, acumulación y metabolismo de triglicéridos, aunque su implicación en otras funciones como órgano productor de sustancias (adipocinas) con acción autocrina, endocrina y paracrina es cada vez más manifiesta.

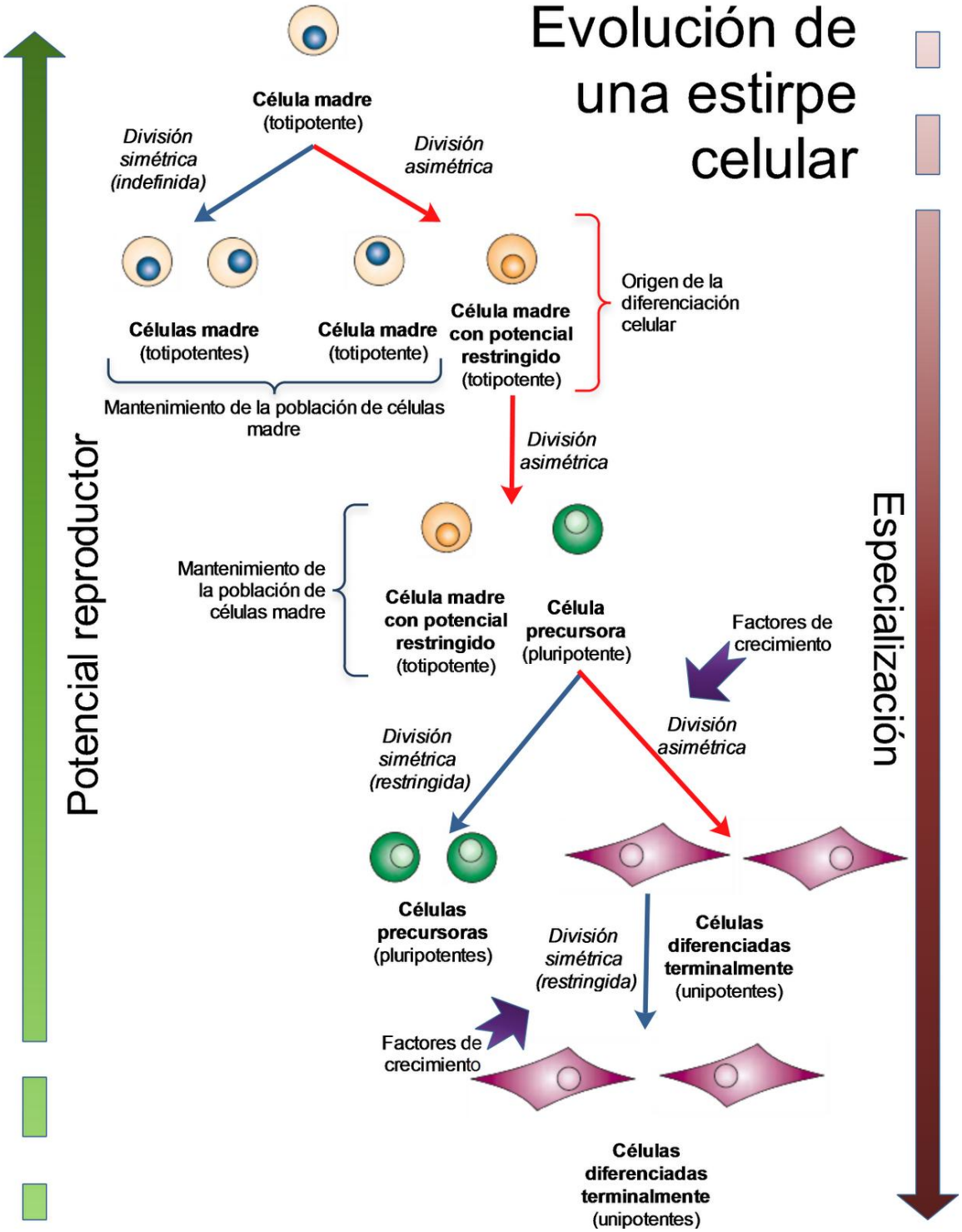
Adipocitos "brite": son considerados un tipo especial de adipocito blanco con la capacidad de expresar el gen *UCP-1*, que hasta hace poco se consideraba específico de los adipocitos pardos, pero que carece de otras características de este tipo de adipocitos (Waldén et al., 2012).

La formación del tejido adiposo blanco comienza antes del nacimiento, aunque la cronología de aparición varía de unas especies a otras. En porcino, la detección de adipocitos comienza al principio del último tercio de gestación (Hausman y Thomas, 1986) y la mayor expansión del tejido adiposo tiene lugar rápidamente tras el nacimiento. Sin embargo, el desarrollo del tejido adiposo es un proceso continuo a lo largo de la vida y depende, entre otros, de factores ambientales. Así, se ha observado que la cantidad de energía de la dieta estimula la diferenciación de nuevas células grasas (Margareto et al., 2001). Además, una vez que estas células se diferencian el proceso es irreversible (Ailhaud y Hauner, 1998). Es por ello imprescindible conocer cómo se produce y regula la adipogénesis en el cerdo con el fin de encontrar nuevos y eficaces mecanismos para modificar la cantidad y la composición de GIM.

1.4.2.1- Diferenciación celular

La diferenciación celular es el proceso por el cual las células se vuelven estructural y funcionalmente distintas entre sí y se convierten en tipos celulares definidos (Wolpert et al., 2015). Atendiendo al grado de diferenciación, dentro de cualquier organismo podemos encontrar células de tres tipos: células madre (las más indiferenciadas), células precursoras y células diferenciadas. Todas ellas son descendientes de células madre embrionarias que, tras sucesivas divisiones van aumentando en su grado de especialización. Las divisiones que tienen lugar dentro del proceso de diferenciación pueden dar lugar a dos células iguales a la original en cuanto a capacidad de división y grado de especialización (división simétrica) o bien, a una célula igual y otra con una menor capacidad de división y una mayor especialización (Lodish et al., 2000), aumentando así el grado de diferenciación. La figura 5 muestra la evolución de una línea celular, desde una célula madre, capaz de dar origen a todas las estirpes celulares, a una célula diferenciada. Estas células muestran una capacidad de división restringida y sólo pueden dar lugar a células de su misma línea.

Figura 5: Evolución de una estirpe celular a lo largo del proceso de especialización o diferenciación.
(Fuente: http://b-log-ia20.blogspot.com.es/2012/03/mas-alla-del-ciclo-celular)

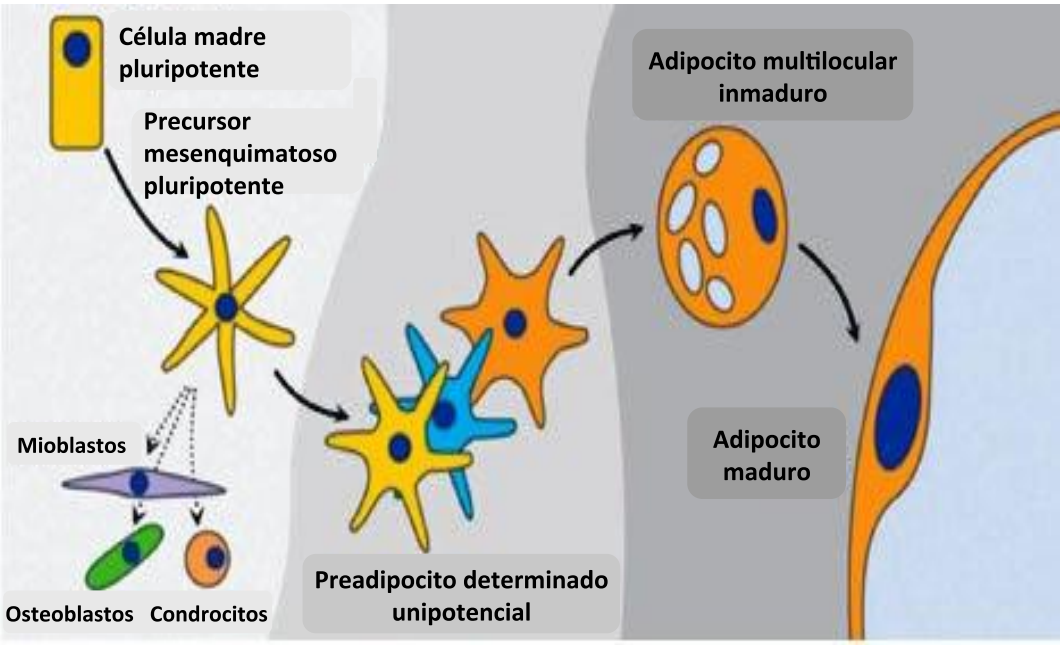


1.4.2.2- Fases de la diferenciación adipocitaria

La formación de una célula adiposa madura sigue el mismo patrón de diferenciación previamente comentado. Partiendo de una célula totipotente y tras una serie de divisiones y de cambios a nivel transcripcional y fenotípico, se genera el adipocito maduro (Figura 6). El estudio de este proceso ha sido posible gracias al uso de modelos celulares *in vitro* (Rosen y MacDougald, 2006) que han permitido la caracterización de los eventos moleculares y celulares que tienen lugar durante la diferenciación de preadipocitos.

Es un proceso minuciosamente regulado, en el que numerosos factores de transcripción regulan positiva o negativamente la expresión de más de 2,000 genes (Farmer, 2006), de forma que se activan aquellos genes característicos de los adipocitos, al mismo tiempo que se reprimen genes inhibitorios de la adipogénesis o que son innecesarios para la función del adipocito.

Figura 6: Esquema de la secuencia de diferenciación del adipocito. (Fuente: Meruane y Rojas, 2010)



Podemos diferenciar dos grandes fases dentro del proceso de adipogénesis, situando a la célula preadipocitaria como nexo de unión:

1. Fase de determinación:

Es la fase inicial en la que la célula totipotente se diferencia a una célula pluripotente, que puede dar lugar a distintas células mesenquimatosas. Una de ellas es el preadipocito, que presenta una

morfología idéntica a la de su precursor pero que ha perdido la capacidad de diferenciarse en distintas líneas celulares (Moreno-Navarrete y Fernández-Real, 2011).

2. Fase de diferenciación terminal:

Esta fase comprende los acontecimientos que tienen lugar en el preadipocito hasta que se convierte en una célula de forma esférica que empieza a acumular lípidos, y que va adquiriendo progresivamente las características morfológicas y bioquímicas propias de los adipocitos maduros. Aunque los fenómenos moleculares implicados en la diferenciación de los adipocitos *in vivo* no son totalmente conocidos, se ha sugerido un modelo basado en la diferenciación de la línea de preadipocitos 3T3-L1:

2.1. Inhibición del crecimiento.

De forma general, el contacto célula a célula entre adipocitos en cultivo favorece la detención del crecimiento celular, si bien no es un prerrequisito imprescindible para que éste se produzca, puesto que en cultivo de preadipocitos primarios de rata en un medio libre de suero y con baja densidad celular, la diferenciación puede ocurrir sin necesidad de dicho contacto (Gregoire et al., 1998).

2.2. Expansión clonal.

Tras la detención del crecimiento, la células deben ser expuestas a una serie de señales adipogénicas y mitogénicas para continuar la diferenciación (Gregoire et al., 1998). Estas señales inducen a las células a reentrar en el ciclo celular, y se produce al menos una replicación de DNA y duplicación celular. Esta expansión mitótica clonal de células comprometidas es esencial para completar la diferenciación terminal en adipocitos maduros (Moreno-Navarrete y Fernández-Real, 2011).

2.3. Cambios tempranos en la expresión de genes.

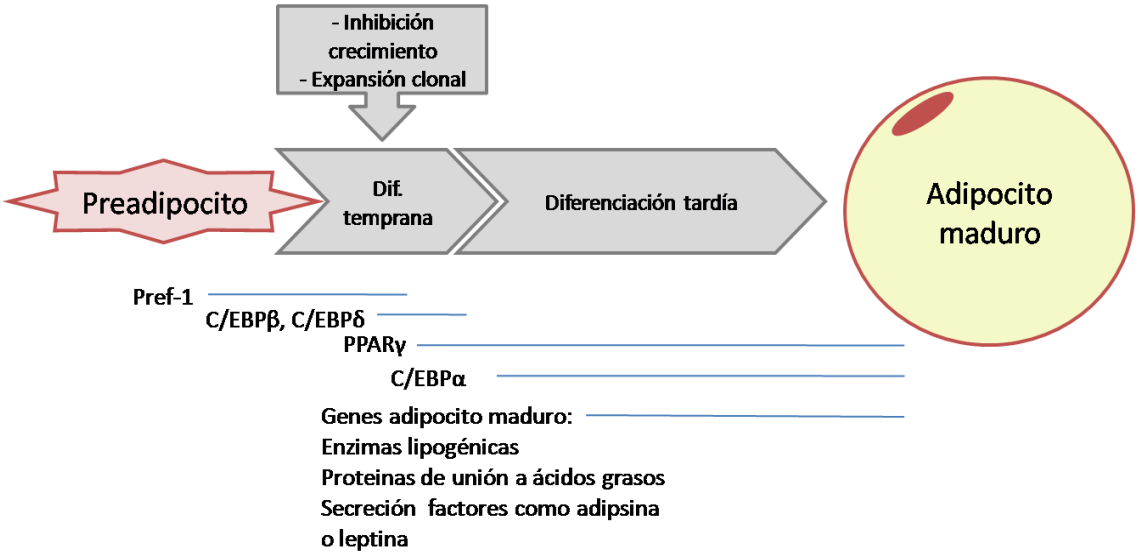
Tras la expansión clonal, se inicia la activación transcripcional de genes específicos del adipocito, que produce una serie de cambios bioquímicos y morfológicos relacionados con la adquisición del fenotipo de adipocito maduro. También se reprimen genes característicos de preadipocito que inhiben la diferenciación, como *DLK1.*

Durante esta fase se produce un aumento en la expresión de los genes *CEBPB* y *CEBPD* que codifican factores de transcripción esenciales en la regulación de la adipogénesis. El factor de transcripción *PPARG* es detectable pero escaso en preadipocitos; sus niveles aumentan rápidamente tras la inducción hormonal a la diferenciación y son fácilmente detectables en el segundo día de diferenciación de adipocitos 3T3-L113 (Gregoire et al., 1998). Posteriormente, se produce una disminución de *CEBPB* y *CEBPD* y la inducción de *CEBPA*, que permanecerá

expresándose hasta el final de la diferenciación (Figura 7). Tanto *PPARG* como *CEBPA* juegan un papel fundamental en la fase de diferenciación tardía de la adipogénesis, ya que regulan la expresión de muchos de los genes característicos del adipocito maduro, como *aP2*, *GLUT4*, *SCD1*, *PEPCK* o *LEP* (Rosen et al., 2000).

Durante la diferenciación también se producen cambios en la morfología celular (perdiendo la forma inicial de fibroblasto hacia una forma esférica, debido al almacenamiento de lípidos en el interior de la membrana celular), en el citoesqueleto y en la matriz extracelular.

Figura 7: Esquema de los genes expresados o reprimidos durante la adipogénesis en las fases de diferenciación temprana y tardía.



2.4. Eventos tardíos y diferenciación terminal.

Durante la fase final de la diferenciación, se observa un incremento en la expresión y actividad de enzimas lipogénicas, tales como FASN, ME, G3PDH, ACACA o SCD1 (Spiegelman et al., 1993). Durante esta etapa aumenta también considerablemente la sensibilidad a la insulina, debido a un gran aumento en el número de receptores de insulina y transportadores de glucosa dependientes de insulina (GLUT4) (Moreno-Aliaga y Martinez, 2002).

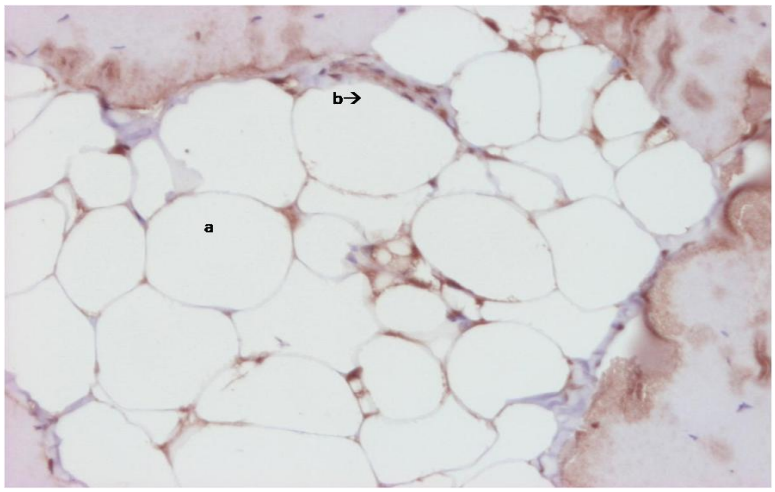
Además de la expresión de genes relacionados con el metabolismo lipídico, las células también sintetizan otros productos considerados específicos del tejido adiposo, como aP2 (una proteína fijadora de ácidos grasos específica de adipocitos) o perilipina, (proteína asociada a las gotas lipídicas), así como sustancias endocrinas y paracrinas (por ejemplo leptina, adipsina y monobutirina) (Gregoire et al., 1998).

Una vez que el tejido adiposo está completamente formado, los adipocitos representan entre uno y dos tercios del mismo. Según la clasificación establecida por Bourin y colaboradores (2013), en el tejido adiposo podemos diferenciar, tras una digestión enzimática, dos grandes grupos celulares:

- Adipocitos maduros (Figura 8)
- Estroma vascular (SVC), que a su vez se compone de
    - Células hematopoyéticas
        - Células madre y precursoras
        - Granulocitos
        - Monocitos
        - Linfocitos
    - Fibroblastos
    - Células endoteliales
    - Pericitos
    - Células estromales derivadas del tejido adiposo (*ASCs*) y que incluye células multipotenciales con capacidad para diferenciarse en distintas líneas celulares, como adipocitos, fibroblastos, etc.

Este precursor embrionario multipotente (*ASCs*) ha sido considerado el origen del tejido adiposo y ha sido denominado "*preadipocito primario*" (Cawthorn et al., 2012), ya que posee capacidad para diferenciarse en células unipotentes y comprometidas hacia el desarrollo de varios tipos celulares determinados, tales como adipocitos, condrocitos, osteoblastos y miocitos (Moreno-Aliaga y Martinez, 2002). Recientemente, los estudios relacionados con *ASCs*, han contribuido a aumentar el conocimiento sobre los procesos celulares relacionados con el compromiso de las células multipotenciales hacia preadipocitos (Cawthorn et al., 2012).

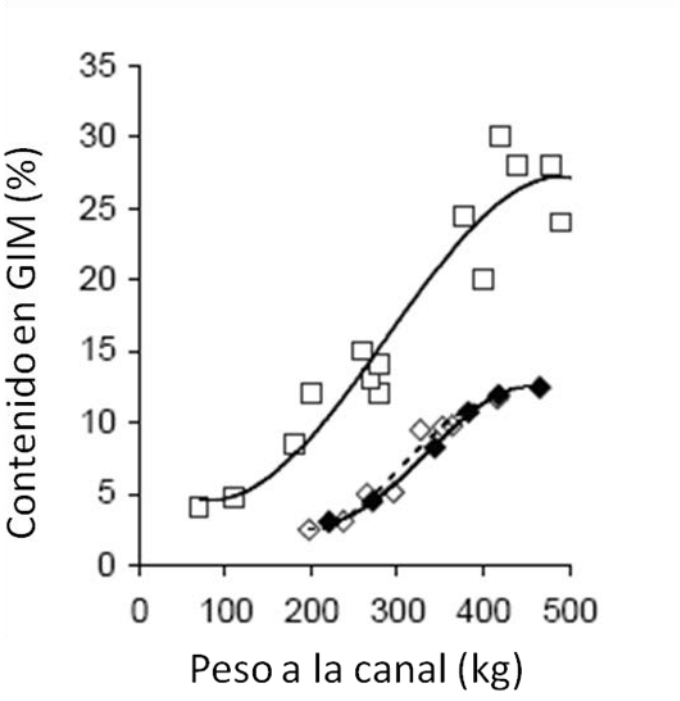
Figura 8: Imagen histológica de células adiposas maduras (a) e inmaduras (b). Tinción Hematoxilina-Eosina con inmunohistoquímica.



1.4.2.3- Particularidades del adipocito muscular

Dentro de los distintos depósitos grasos cabe destacar la grasa subcutánea por ser el depósito más extenso y la GIM por su papel en la calidad de la carne previamente comentado. Los preadipocitos y adipocitos encontrados en ambos depósitos muestran diferencias importantes en cuanto a los procesos de adipogénesis y lipogénesis. En primer lugar, los preadipocitos musculares comienzan el proceso de diferenciación de una forma más tardía que los de la grasa subcutánea, tanto en cultivos celulares, tratados con inductores de la diferenciación (Wang et al., 2013), como *in vivo* (Gondret et al., 2008). Se ha observado que el crecimiento del depósito intramuscular en vacuno sigue el patrón representado en la figura 9.

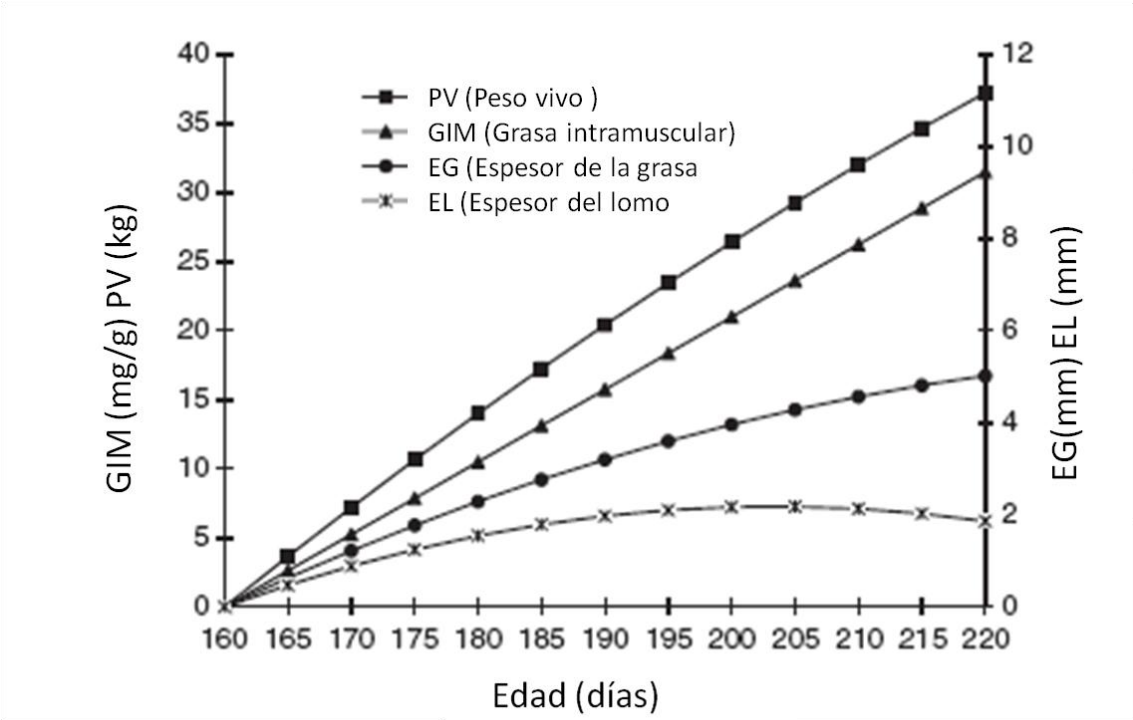
Figura 9: Relación entre la cantidad de grasa intramuscular y el peso de la canal en tres cruces de vacuno de carne con distintos potenciales de engrasamiento. (Fuente: Pethick et al., 2006)



Además, Pethick y colaboradores (2006) han sugerido que en animales con escaso potencial adipogénico y gran desarrollo muscular, estas curvas podrían estar desplazadas hacia la derecha de la gráfica y que, por lo tanto, en pesos habituales de sacrificio los animales no llegarían a alcanzar la fase lineal de aumento de la GIM. Esta hipótesis es coherente con los resultados observados en razas modernas de cerdo, en las que la GIM no aumenta a lo largo de un amplio rango de pesos a la canal (Dunshea y D'Sousa, 2003). Por otro lado, se ha observado que en Duroc, una raza más grasa que las razas de cerdo blanco, los animales de entre 5 y 7 meses de edad aproximadamente, muestran un crecimiento lineal del contenido en GIM, mientras que el incremento de los depósitos

subcutáneos disminuye entre estas dos edades (Bosch et al., 2012) (Figura 10). Estos resultados sugieren una distinta regulación, no sólo del proceso de adipogénesis, sino también de lipogénesis que tienen como resultado patrones de crecimiento diferentes en ambos depósitos grasos.

Figura 10: Relación entre cantidad de grasa intramuscular y edad en cerdos de raza Duroc. (Fuente: Bosch et al., 2012)



Más allá de las diferencias relativas a la diferenciación adipocitaria, también se han encontrado diferencias en el metabolismo lipídico entre adipocitos musculares y de la grasa subcutánea. Así, se ha observado que los adipocitos musculares tienen preferencia por el uso de la glucosa para la síntesis lipídica, mientras que los adipocitos de la grasa subcutánea utilizan principalmente ácidos grasos durante la lipogénesis (Wang et al., 2013). Por último, se ha descrito que los adipocitos localizados en el depósito subcutáneo acumulan más lípidos y de forma más rápida que los adipocitos musculares (Zhou et al., 2010; Kouba y Mourot, 2011).

1.4.2.4- Regulación transcrpcional de la adipogénesis

1.4.2.4.1- Cascada de activación de factores de transcripción

Debido al interés que la adipogénesis suscita, principalmente desde el punto de vista de la salud humana, los estudios que abordan la regulación de la misma son abundantes. Como se ha comentado anteriormente, numerosos mecanismos celulares y moleculares controlan el proceso de

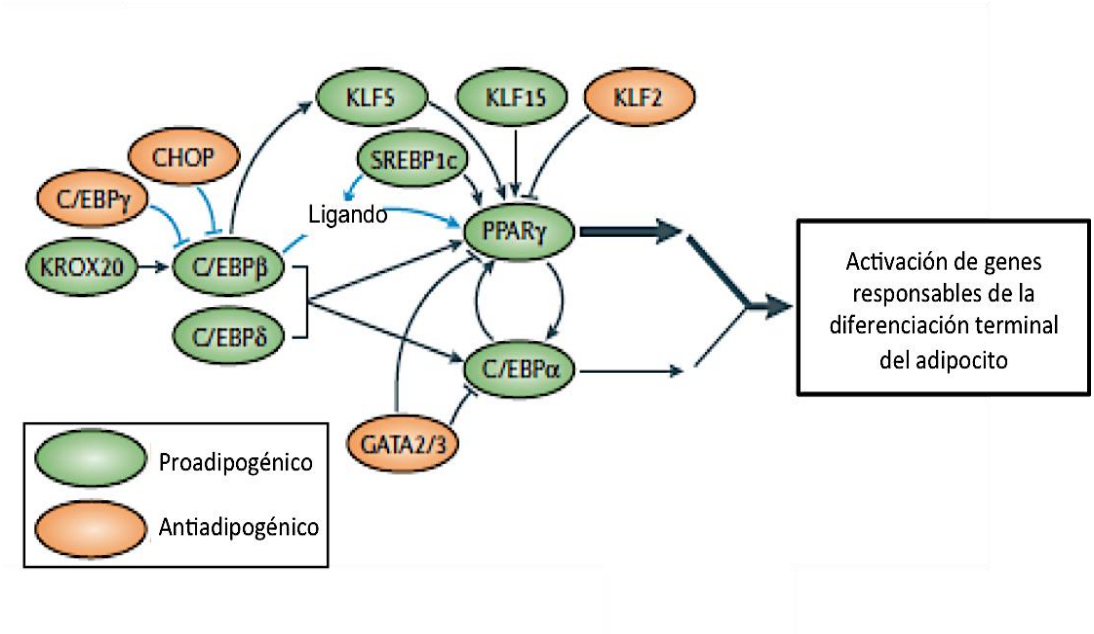
diferenciación adipocitaria, de forma que los factores de transcripción se activan o reprimen unos a otros de forma secuencial (Ma et al., 2015).

Dentro de los factores de transcripción involucrados en la regulación de la adipogénesis, la familia CEBP y la familia PPAR son cruciales y han sido ampliamente estudiados (Rosen y MacDougald, 2006), sin embargo, actualmente el conocimiento sobre estos mecanismos transcripcionales es más profundo y se conocen nuevos reguladores que también serán mencionados brevemente.

La familia CEBP está constituida por varias isoformas, entre ellas, *CEBPA, CEBPB, CEBPG, CEBPD* y *CHOP* se expresan en adipocitos. La activación o inhibición de estos genes sigue una secuencia temporal en la que la expresión temprana de *CEBPB* y *CEBPD* conduce a la expresión, ya en la fase tardía de *CEBPA* (Rosen y MacDougald, 2006) (Figura 6). El gen *CEBPB* es crucial para el desarrollo de la adipogénesis en líneas inmortalizadas de preadipocitos, sin embargo, su papel parece no ser tan relevante en cultivos de fibroblastos. Por otro lado, se ha observado que animales con este gen reprimido (*CEBPB*$^{-/-}$) presentan una adiposidad reducida (Tanaka et al., 1997), pero se desconoce si es a causa de una alteración en la adipogénesis o en el metabolismo lipídico. Este factor de transcripción, junto con *CEBPD* es el encargado de activar la expresión de *CEBPA* y *PPARG* (Figura 11).

CEBPA parece ser un factor nuclear indispensable y crítico en el proceso de diferenciación de los adipocitos (Yeh et al., 1995) que induce la expresión de forma directa de numerosos genes específicos del adipocito maduro, en los cuales se han identificado lugares de unión a *CEBPA* en las regiones promotoras (MacDougald y Lane, 1995). Además, sus efectos van más allá de lo observado en cultivos celulares, puesto que ratones con este gen reprimido (*CEBPA*$^{-/-}$) carecen totalmente de tejido adiposo (excepto en la glándula mamaria) (Linhart et al., 2001). Varios estudios han puesto de manifiesto que este factor de transcripción es no sólo necesario, sino también suficiente, para poner en marcha el proceso de diferenciación de los adipocitos incluso en ausencia de agentes inductores de la diferenciación (Freytag et al., 1994). En concordancia, se ha observado que la supresión de la expresión de *CEBPA* en cultivo celular provoca una inhibición en la diferenciación terminal de los adipocitos, lo cual parece indicar que este proceso requiere el mantenimiento de la expresión sostenida de *CEBPA* (Lin y Lane, 1992). Finalmente otros miembros de la familia CEBP como *CHOP* y *CEBPG*, parecen suprimir la adipogénesis, probablemente debido a una inactivación por herterodimerización con *CEBPB* (Darlington et al., 1998).

Figura 11: Genes involucrados en la cascada de activación de la adipogénesis en la fase inicial. (Fuente: Adaptado de Rosen y MacDougald, 2006)



A pesar de la importancia de la familia CEBP en la regulación de la adipogénesis, estos factores de transcripción son claramente insuficientes para activar el proceso de diferenciación en ausencia de *PPARG*, el cual fue relacionado por primera vez con la adipogénesis a principios de los años noventa y es actualmente considerado como el "gran director" de la adipogénesis. Es el único miembro de una familia de receptores nucleares/factores de transcripción (PPAR), que se encuentra expresado en altos niveles específicamente en tejido adiposo. La expresión de *PPARG* antecede la inducción de *CEBPA* en la cascada de eventos que conducen a la diferenciación de los adipocitos (Figuras 7 y 11). Al igual que lo observado con *CEBPA*, la expresión retroviral de *PPARG* es suficiente para inducir la conversión de varias líneas celulares de fibroblastos en adipocitos (Tontonoz et al., 1994). En estudios *in vivo*, la ausencia total de tejido adiposo en animales *PPARG*$^{-/-}$, posicionan a este factor de transcripción y al receptor nuclear *RXRA*, con quien debe formar necesariamente heterodímeros, como factores de transcripción imprescindibles en la regulación de la expresión génica que desencadena la diferenciación adipocitaria (Rosen et al., 1999). Los factores de transcripción *CEBPB* y *CEBPD* parecen jugar también un importante papel en la inducción de *PPARG*. De hecho, su expresión ectópica provoca un incremento en los niveles de *PPARG* equivalente al de las células adiposas normales (Wu et al., 1995). Además de la regulación transcripcional, algunos estudios relacionan la expresión de *PPARG* con los niveles de lípidos en el organismo, destacando su función en el mantenimiento de la homeostasis lipídica (Morrison y Farmer, 2000) y no sólo como regulador de la adipogénesis.

Otros factores de transcripción, quizás menos conocidos pero que juegan también un papel importante en el proceso de adipogénesis son KLFs, *SREBP1C*, *STAT5*, LXRs, *EPAS1* o *BMAL1.* La familia KLF está relacionada con la regulación de la apoptosis, la proliferación y la diferenciación. Algunos miembros se expresan en tejido adiposo, desempeñando el papel de activadores (*KLF15, KLF5* o *KLF6*, que actúan favoreciendo la expresión de *GLUT4*, activando el promotor de *PPARG2* o inhibiendo la expresión de *DLK1*, un potente inhibidor de la adipogénesis, respectivamente), mientras que otros actúan como factores antiadipogénicos (*KLF2*, que inhibe el promotor de *PPARG2* y *KLF7*) (Rosen y MacDougald, 2006).

Otro factor que también parece estar implicado en el proceso de diferenciación es *SREBP1C*. La coexpresión de este factor de transcripción incrementa la actividad transcripcional de *PPARG* incluso en ausencia de sus ligandos activadores (Kim y Spiegelman, 1996). Curiosamente, estudios *in vivo* con ratones *SREBP1C*$^{-/-}$ han demostrado que éstos desarrollan todos los depósitos grasos con normalidad (Shimano et al., 1997); *SREBP1C* es el factor de transcripción que presenta más inconsistencias entre estudios *in vivo* e *in vitro*.

La familia STAT está compuesta por proteínas citoplasmáticas que modifican la expresión génica en respuesta a señales extracelulares unidas a los receptores JAK (Darnell, 1997). Los miembros *STAT1*, *STAT5A* y *STAT5B* se encuentran sobreexpresados durante la diferenciación de adipocitos en cultivo y, aunque se desconoce exactamente el mecanismo de acción, se ha observado que ratones -/- para estas dos isoformas presentan una menor cantidad de tejido adiposo blanco, lo que demuestra un papel importante durante la adipogénesis (Morrison y Farmer, 2000).

El papel de los receptores nucleares LXR en el control de la adipogénesis es controvertido, puesto que diferentes estudios han revelado efectos positivos, negativos y neutros. Sin embargo, su papel en la regulación de la fisiología del adipocito maduro sí está más clara (Rosen y MacDougald, 2006).

Finalmente *EPAS1, CREB* y *BMAL1* han sido caracterizados como promotores de la adipogénesis (Rosen y MacDougald, 2006), mientras que distintos miembros de la familia GATA, entre ellos *GATA2* y *GATA3* inhiben la adipogénesis (Figura 11), favoreciendo el desarrollo de otros tipos celulares, como las células sanguíneas (Rosen y MacDougald, 2006).

Dentro de la serie de acontecimientos moleculares que tienen lugar durante la adipogénesis, hay también genes cuya expresión se suprime. El más conocido es *DLK1*, que codifica para una proteína transmembrana. El gen *DLK1* presenta altos niveles de expresión en preadipocitos y su expresión disminuye durante la diferenciación, siendo completamente indetectable en adipocitos maduros (Smas y Sul, 1996).

1.4.2.4.2- Factores que estimulan la adipogénesis

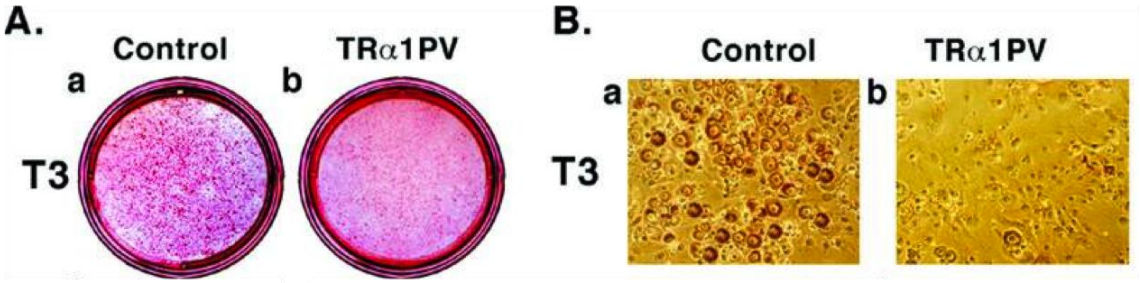
La adipogénesis, además de estar sometida a una regulación intrínseca, dependiente de los factores de transcripción, necesita de señales extracelulares que desencadenen la cascada de cambios en la transcripción génica. En general, como ocurre en todas las células, el crecimiento y la diferenciación están controlados por la comunicación célula – célula y entre células y la matriz extracelular. Las proteínas de la matriz extracelular juegan un papel importante en la regulación de la diferenciación, puesto que están relacionadas con los cambios extracelulares que tienen lugar y permiten la expresión de genes involucrados en la diferenciación.

Algunas de las señales adipogénicas extracelulares más conocidas son la insulina, el factor de crecimiento IGF-1, las hormonas tiroideas, los estrógenos, los gluco y mineralocorticoides y los agonistas de *PPARG* (Moreno-Navarrete y Fernández-Real, 2011). Estas hormonas y factores de crecimiento actúan mediante receptores celulares, transmitiendo señales externas de crecimiento y diferenciación a través de cascadas de señalización intracelulares. Por ejemplo, para que la diferenciación de los adipocitos comience es necesaria una señal hormonal que la estimule. Aunque el espectro completo de agentes inductores de la adipogénesis varía con cada cultivo celular, insulina, IGF-1 y glucocorticoides son generalmente considerados necesarios para la diferenciación. La insulina forma parte de una cascada de señalización crucial para el correcto desarrollo de la adipogénesis; así, la ausencia de las proteínas de sustrato del receptor de insulina (IRS), conlleva la inhibición de la adipogénesis (Blüher et al., 2002).

El factor de crecimiento IGF-1 estimula la adipogénesis en cultivo primarios de preadipocitos de distintas especies, entre ellas el cerdo (Ramsay et al., 1989), siendo un regulador imprescindible en la diferenciación de células adiposas.

La hormona tiroidea T3 tiene un papel central en el metabolismo y la homeostasis energética. Además, T3 estimula la adipogénesis en cultivos celulares de preadipocitos mediante su unión al receptor tiroideo TRα1, como se ve en la figura 12.

Figura 12: Cultivo celular de células control (TRa1) y mutadas para el receptor TRa1 (TRa1PV). (Fuente: Ying et al., 2007)



Imágenes tomadas tras la inducción a la adipogénesis. Las gotas lipídicas han sido teñidas mediante Oil Red-O (A) o evidenciadas mediante microscopía de contraste de fases (B). En las muestras control, "a", se observa una mayor acumulación de lípidos, consecuencia de una correcta

adipogénesis en estas células, mientras que en las células TRα1PV (b), que presentan una mutación que impide la unión del receptor TRα1 con T3 se observa la ausencia de acumulación lipídica.

Los glucocorticoides (GC) son también potentes inductores de la adipogénesis in vitro y el hipercortisolismo ha sido asociado con obesidad en la especie humana (Joyner et al., 2000). De hecho, hay receptores para GC en las células preadiposas humanas y se ha observado que los GC activan la expresión de los factores de transcripción *CEBPD* y *PPARG* (Wu et al., 1996), antes mencionados.

Recientemente se ha reconocido la capacidad adipogénica de ciertos factores de crecimiento de fibroblastos (FGF), aunque hace unos años se pensaba que tenían el efecto contrario (Rosen y MacDougald, 2006). En concreto, *FGF1, FGF2* y *FGF10* (este último relacionado con la activación de *CEBPB* según Sakaue y colaboradores (2002)) han sido positivamente relacionados con el proceso de diferenciación y su supresión inhibe la diferenciación (Hutley et al., 2004). Además, FGF1 es una sustancia liberada al medio por las células endoteliales de los microvasos sanguíneos (Hutley et al., 2004), lo que refleja la estrecha relación entre la microvascularizacion y el desarrollo del tejido adiposo.

Por último, recientemente ha aumentado el reconocimiento de la influencia de los factores medioambientales sobre la adipogénesis (Grun y Blumberg, 2006; Sargis et al., 2010; Moreno-Navarrete y Fernández-Real, 2011).

1.4.2.4.3- Factores que inhiben la adipogénesis

Se han descrito numerosos factores inhibidores de la adipogénesis, que pueden actuar bien mediante sus propiedades mitogénicas (Gregoire et al., 1998), como es el caso de numerosos factores de crecimiento, o bien mediante mecanismos independientes, como sucede con *DLK1*.

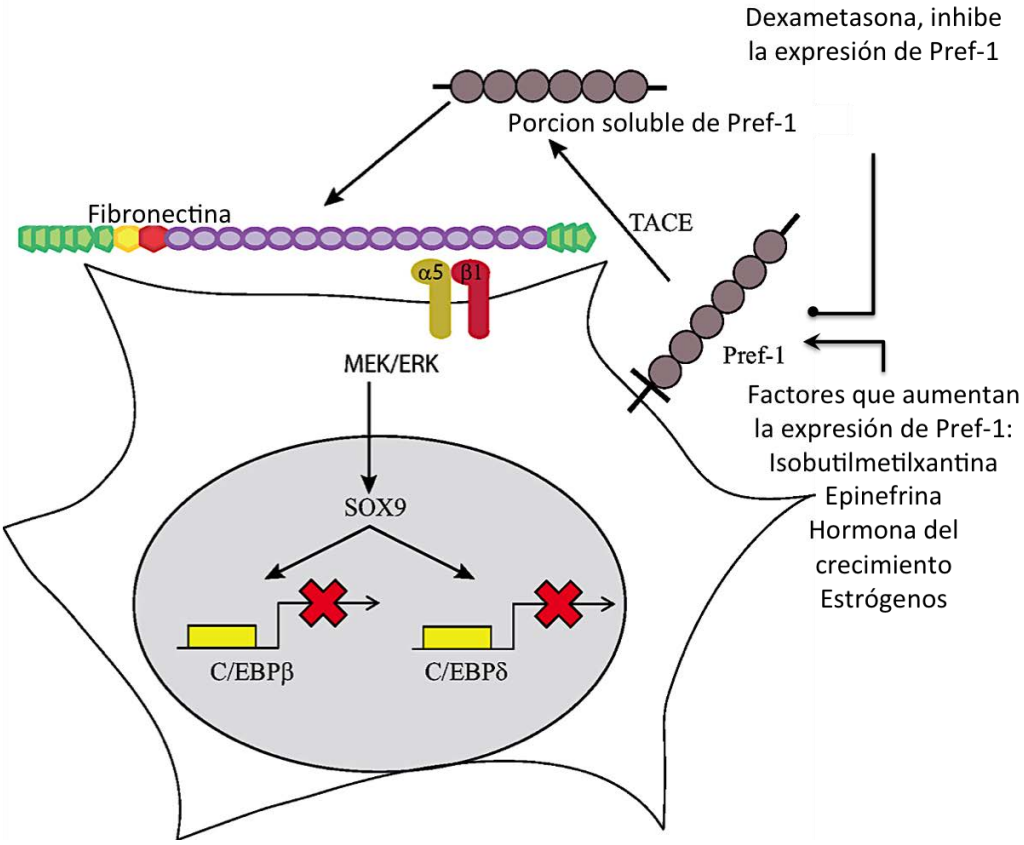
Algunos factores inhibidores de la diferenciación son las glicoproteínas Wnt, TGFβ, citoquinas proinflamatorias tales como IL-1 o TNFα, IFNG, $PGF_{2\alpha}$ o TPA (Gregoire et al., 1998; Moreno-Navarrete y Fernández-Real, 2011). Sin embargo, esta clasificación no siempre es exacta y sencilla, puesto que, en muchas ocasiones, la actividad del agente depende de la dosis, el momento en el que se añada al cultivo, el tipo de cultivo (cultivo primario o células preadiposas), el medio de cultivo (con o sin suero), etc. Así pues, numerosos compuestos han sido descritos como inductores e inhibidores. Algunos de estos compuestos son el ácido retinoico (AR) (su acción es dependiente de la concentración, a dosis bajas estimula la diferenciación, mientras que dosis altas la inhibe (Suryawan y Hu, 1997; Bonet et al., 2003)), GH (en cultivos primarios), PDGF o FGF.

La familia de glicoproteínas Wnt bloquea completamente la inducción de algunos de los genes clave en la cascada de la adipogénesis, como son *CEBPA* y *PPARG* (Moreno-Navarrete y Fernández-Real, 2011).

Otro importante inhibidor de la adipogénesis pertenece a la familia TGF; en concreto, *TGFβ*, que se expresa en cultivos de adipocitos y que, a pesar de estimular la proliferación de preadipocitos, inhibe su posterior diferenciación bloqueando a los factores de transcripción CEBP, principalmente *CEBPB* y *CEBPD* (Choy y Derynck, 2003).

La proteína transmembrana DLK1/PREF-1 es activada mediante escisión proteolítica que libera la porción extracelular, lo cual produce una inhibición de la adipogénesis (Figura 13). La expresión forzada de esta proteína en cultivos de preadipocitos detiene la adipogénesis y ratones *DLK1*$^{-/-}$ presentan un retraso en el crecimiento acompañado de una adiposidad aumentada y precoz (Moon et al., 2002), mientras que la expresión aumentada de este gen se ha relacionado con lipoatrofia (Lee et al., 2003); estas evidencias lo posicionan como un potente inhibidor de la adipogénesis.

Figura 13: Inhibición de la adipogénesis por *DLK1/Pref-1*. (Fuente: Adaptado de Hudak y Sul, 2013)



Pref-1 es una proteína transmembrana que se escinde mediante la acción de la enzima convertidora de TNFα (TACE), lo que genera una fracción soluble que interactúa con la fibronectina activando la ruta MEK/ERK. Esta ruta aumenta los niveles de *SOX9,* factor de transcripción que al unirse a las regiones promotoras de *CEBPB* y *CEBPD* bloquea su expresión y, por lo tanto, la diferenciación de adipocitos.

Las citoquinas proinflamatorias inhiben la adipogénesis en cultivos de preadipocitos reduciendo la expresión de *PPARG* y de *CEBPA* por un lado, y bloqueando la acción de la insulina por otro (Moreno-Navarrete y Fernández-Real, 2011).
Por último, ciertos fármacos o compuestos químicos como los fármacos antirretrovirales o la metformina, utilizada en el tratamiento de la diabetes tipo 2 también inhiben la diferenciación de preadipocitos (Leow et al., 2003; Alexandre et al., 2008).

1.4.3- Factores que influyen en la cantidad y composición de la grasa intramuscular

La GIM es la acumulación de células grasas entre las fibras musculares. Su contenido y composición dependen de numerosos factores, entre los que se encuentran el desarrollo adipocitario en el interior de los distintos grupos musculares, la síntesis y degradación de ácidos grasos y triacilgliceroles, el almacenamiento de lípidos, etc.
Debido a la importancia de la GIM en la calidad de la carne, se han realizado numerosos estudios para determinar los factores que influyen en su contenido. A continuación, se presentan los factores más importantes:

1.4.3.1- Genética

Diferencias raciales

La raza, y por lo tanto la genética, ha sido destacada como la principal causa que determina la cantidad de GIM en el cerdo, con grandes diferencias entre razas e incluso entre variedades o estirpes (Muriel et al., 2004). Las razas porcinas actuales han sido seleccionadas atendiendo a distintos criterios; en general, se han producido razas con una mayor eficiencia alimentaria (ganancia media diaria e índice de conversión), reproductiva (número de lechones por cerda) y de acumulación de proteína. La selección aplicada ha dado lugar cambios fenotípicos muy marcados. Generalmente, las razas comerciales se han seleccionado para obtener una carne más magra que satisface los gustos del consumidor, ya que le atribuye características más saludables. En consecuencia, esta selección ha producido un descenso marcado en el contenido de GIM en las líneas modernas. Por el contrario, en razas menos seleccionadas este parámetro es muy variable, encontrando desde razas poco engrasadas, como el Tarmworth, hasta razas con un alto nivel de infiltración grasa, como es el caso del cerdo ibérico (Tabla 3).

Tabla 3: Resumen de valores de grasa intramuscular descritos en la bibliografía para distintas razas y estirpes porcinas en los músculos *Longissimus dorsi* (LD) y *Masseter* (MS) (en estirpes de cerdo ibérico).

	Razas modernas (generalmente magras)				Razas no seleccionadas	
Raza	Large White	Landrace	Pietrain	Duroc	Berkshire	Tarmworth
GIM (%)	0.9 - 1.22	1.04	0.81	1.77 - 4.25	2.05 - 3.18	1.2
	(Larzul et al., 1997; Wood et al., 2004)	(Nechtelberger et al., 2001)		(Suzuki et al., 2003; Wood et al., 2004)	(Wood et al., 2004)	

	Estirpes de cerdo ibérico			
Músculo	Entrepelado	Lampiño	Retinto	Torbiscal
MS	4.39%	4.55%	4.46%	2.39%
LD	5.11%	4.84%	4.80%	3.67%
	(Muriel et al., 2004)			

Genes implicados en la cantidad de grasa intramuscular

El contenido en GIM está potencialmente regulado por numerosos genes y rutas metabólicas. Algunos de los genes candidato que podrían estar relacionados con la GIM se especifican a continuación:

a) Genes candidato relacionados con el desarrollo adipocitario:
Son, principalmente, factores de transcripción que regulan el desarrollo y la diferenciación de adipocitos. Algunos de los genes más importantes en este grupo han sido mencionados en el apartado 1.4.2.2: *PPARG, RXRG, RARA, CEBPA, CEBPB, ASXL2, DLK1, EGR2, KLF5* (Tontonoz et al., 1994; Darlington et al., 1998; Rosen et al., 2002; Chen et al., 2005; Oishi et al., 2005; Park et al., 2011).

b) Genes candidato relacionados con la síntesis y metabolismo de ácidos grasos:
Se expresan en el adipocito maduro, su expresión regula los procesos de lipogénesis y lipolisis y por lo tanto, influye sobre el contenido lipídico y el volumen de los adipocitos*: SREBP1C, ACACA, FASN, SCD1, ME1, ELOVL6, FABP4, DGAT, CPT-1, AdPLA, ACSL4, ACOX1* (Singh et al., 1992; Nechtelberger et al., 2001; Cronan y Waldrop, 2002; Horton et al., 2002; de Sousa et al., 2005; Damon et al., 2006; Durgan et al., 2006; Kim et al., 2011; Corominas et al., 2013b; Skiba et al., 2013; Tuohetahuntila et al., 2015).

c) Genes candidato relacionados con el control de la homeostasis energética y la ingesta voluntaria:
Se expresan principalmente en el hipotálamo, considerado el centro regulador de la homeostasis energética en el organismo. La activación del gen *LEPR* da lugar a una cascada de señalización que modula la expresión en el hipotálamo de ciertos neuropéptidos relacionados con la ingesta voluntaria y gasto energético (Óvilo et al., 2010). Entre los genes regulados por *LEPR* en esta cascada de señalización cabe destacar*: MC4R, POMC, NPY, AGRP y CART* (Kristensen et al., 1998; Elias et al., 1999; Korner et al., 2001; Balthasar et al., 2004) entre otros. La activación del gen *LEPR* depende del tejido graso, encargado de regular la ingesta de alimentos, mediante la expresión del gen *LEP*, que tiene lugar en los adipocitos cuando estos tienen niveles altos de lípidos acumulados (revisado en (Harris, 2014)), y que es la hormona responsable de inhibir el apetito a nivel del SNC, mediante su unión al receptor. Este mecanismo de regulación de la ingesta, sin embargo, parece estar alterado en el caso del cerdo ibérico, que presenta altos niveles de leptina circulante conjuntamente con elevado apetito (Fernández-Figares et al., 2007). Esta alteración podría estar relacionada con la mutación en el gen *LEPR* explicada previamente, que está asociada a una reducción de su expresión a nivel hipotalámico, y de la de otros neurotransmisores de la ruta (Óvilo et al., 2010).

d) Genes candidato relacionados con el metabolismo de la glucosa:
La regulación del metabolismo de la glucosa tiene también una importante relación con el control de la homeostasis energética (Könner et al., 2009). Algunos de los genes relacionados con este proceso son: *IGF1, INS, INSR, GLUT, G6PC o PCK2* (Garcia de Herreros y Birnbaum, 1989; Ramsay et al., 1989; Beale et al., 2007; Liu et al., 2008; Könner et al., 2009).

1.4.3.2- Epigenética

Es la ciencia que estudia los cambios heredables en la función de los genes que no entrañan una modificación en la secuencia del material genético (Morris, 2001).
En los últimos años, esta rama de la genética ha cobrado gran importancia, se están dedicando grandes esfuerzos a comprender los posibles cambios que, sin afectar a la estructura del ADN, pueden afectar al fenotipo del organismo. Los mecanismos por los que estos cambios tienen lugar son variados, y pueden ser clasificados en tres grandes grupos:

- Metilación del ADN, el mecanismo más conocido (Goldberg et al., 2007). Consiste en la adición de un grupo metilo en los residuos C de los dinucleótidos C-G, provocando un cambio estructural que afecta a la transcripción del ADN. Normalmente, la metilación se produce en zonas ricas en dinucleótidos C-G, llamadas islas CpG.

- Modificaciones en la cromatina, que pueden a su vez ser consecuencia de cambios covalentes en las histonas (Wang et al., 2007), por ejemplo acetilación, fosforilación o metilación de las histonas que tienen como consecuencia cambios en la estructura de la cromatina. También se han descrito en la literatura modificaciones no covalentes, como es el caso de la remodelación de la cromatina (se cree que se produce un cambio en la condensación de la cromatina mediante alteración de las uniones histona-ADN), o la incorporación de variantes histónicas especializadas (Goldberg et al., 2007).
- ARN no codificante: se ha observado que ciertos tipos de ARN no codificante pueden actuar, en colaboración con los procesos anteriormente descritos provocando cambios estables que pueden ser heredados en las sucesivas divisiones celulares (Goldberg et al., 2007).

Todos estos mecanismos tienen además rutas de acción interrelacionadas (Goldberg et al., 2007).
Un ejemplo de regulación epigenética podría ser el cambio fenotípico observado tanto en personas como en modelos animales cuyas madres han sufrido escasez de nutrientes durante la gestación (Burdge et al., 2007). En el cerdo ibérico, este fenómeno ha sido recientemente estudiado (Ovilo et al., 2014c). Se evaluó la expresión de distintos genes a nivel hipotalámico, en lechones nacidos de cerdas con alimentación adecuada y restringida durante la gestación, los genes *LEPR* y *POMC*, intermediarios en la ruta de señalización anorexigénica de la leptina, presentaron niveles de expresión menores en las hembras nacidas de madres restringidas. La importancia de este fenómeno desde el punto de vista productivo, implica un mayor desarrollo del contenido graso en estos cerdos, así como un crecimiento compensatorio experimentado por los individuos cuyas madres sufrieron una restricción alimenticia severa. Por otro lado, se ha observado que animales adultos procedentes de madres con distintos planos de alimentación depositan cantidades diferentes de GIM y que además, la composición de esta GIM también varía en función de la alimentación de la madre (Barbero et al., 2013).

1.4.3.3.- Sexo

Hay resultados contradictorios en los trabajos realizados en la especie porcina sobre el efecto del sexo en el contenido en GIM. Algunos de ellos (Latorre et al., 2003a; Correa et al., 2006) reportan un efecto significativo del sexo sobre este parámetro mientras que otros (Cisneros et al., 1996b; Hamilton et al., 2000; Latorre et al., 2004) no detectan ningún efecto. En un trabajo reciente, se observó que los machos castrados presentan un porcentaje de GIM superior a las hembras (2,49% vs 2,00%) (Bahelka et al., 2007) coincidiendo con resultados encontrados por otros autores (Correa et al., 2006; Latorre et al., 2003a; Serrano et al., 2009).

También se ha observado una mayor cantidad de ácidos grasos saturados (AGS) y una menor cantidad de ácidos grasos poliinsaturados (AGPI) en machos castrados respecto a hembras en cerdo ibérico (Serrano et al., 2009) y en un cruce de Hampsire (Nilzén et al., 2001).

1.4.3.4- Edad

Existe una relación directa entre la edad y cantidad de GIM, ya que es en estadios más tardíos del desarrollo cuando los depósitos grasos aumentan. Este hecho, aunque está bien documentado (Gerbens, 2004; Bosch et al., 2012), no siempre fue observado en trabajos previos (Ellis et al., 1996; Lo Fiego et al., 2010). Por otro lado, varios estudios (Latorre et al., 2004; Correa et al., 2006; Bahelka et al., 2007) no encontraron diferencias significativas en el contenido en GIM en cerdos sacrificados a distintas edades. La variabilidad en los resultados encontrados puede deberse, por un lado, al material animal utilizado (genotipo, sexo, peso), y por otro, al diseño experimental, transversal en la mayoría de los casos y longitudinal en el trabajo realizado por Bosch y colaboradores (2012). Este diseño realizado por Bosch y colaboradores permite, mediante el uso de medidas repetidas, conocer la evolución de la GIM a lo largo de la vida de cada animal. En cuanto a la composición de los ácidos grasos en la GIM, se ha observado que con la edad se produce un aumento de los AGMI en detrimento de los AGPI, mientras que los AGS permanecen estables (Lo Fiego et al., 2010; Bosch et al., 2012).

1.4.3.5- Sistema de producción

A su vez engloba distintos parámetros, como son el ambiente, el ejercicio y la alimentación (Cava et al., 1999; Edwards, 2005; Rey et al., 2006a; López-Bote et al., 2008). El sistema más extendido en producción porcina es el sistema intensivo. En el caso del porcino ibérico, el panorama es más complejo, puesto que, como se ha comentado anteriormente, hay distintos sistemas productivos aceptados. El sistema de producción ha demostrado tener efecto sobre el perfil de ácidos grasos de la GIM, así, se ha observado que los cerdos alimentados en montanera presentan un perfil más rico en C18:1 n-9, C18:3 n-3 y AGMI, con un menor contenido en AGS que los alimentados en intensivo (Cava et al., 1997; Ruiz et al., 1998; Andrés et al., 2001; Tejerina et al., 2012). Este perfil refleja la composición de ácidos grasos presente en las bellotas, junto con el alto contenido en C18:3 n-3 de la hierba (Rey y López-Bote, 2001; Rey et al., 2006b). Por otro lado, los animales de recebo presentan valores intermedios (Ruiz et al., 1998; Andrés et al., 2001; Tejerina et al., 2012). Los resultados obtenidos al evaluar el contenido en GIM son variables, encontrando trabajos que describen un efecto positivo de la producción en montanera sobre el contenido de GIM (Andrés et al., 2001; Ventanas et al., 2007) mientras que en otros casos, los resultados no muestran diferencias significativas (Carrapiso y García, 2005; Tejerina et al., 2012). Este hecho puede ser debido a la

formulación de piensos con un contenido en ácidos grasos y nutrientes similares a los disponibles en la alimentación en montanera (Tejerina et al., 2012).

1.4.3.6- Alimentación

Pese a que la alimentación forma parte del sistema de producción, debido a su importancia, es tratada de forma independiente. Además, las estrategias nutricionales para modificar el contenido en GIM son muy diversas y pueden ser utilizadas tanto de forma prenatal como postnatal.

1.4.3.6.1- Alimentación prenatal

Como se ha mencionado anteriormente, es posible afectar los parámetros productivos así como de composición de la canal en animales modificando la dieta de las madres durante la gestación. Por ejemplo, se ha observado que la adición de grasa en las dietas de gestación y lactación aumenta el número de células adiposas en la grasa dorsal y en el lomo de los lechones al nacimiento, aumentando por tanto el potencial de acumulación de grasa en estos tejidos (Mourot, 2001).
Por otro lado, el nivel energético de la dieta durante la gestación también afecta al desarrollo fetal y postnatal del animal. Los fetos expuestos a un déficit nutricional, desarrollan una serie de adaptaciones fisiológicas y metabólicas (conocidos como procesos de programación prenatal) con el fin de adaptarse y sobrevivir en un entorno de recursos limitados y de mala nutrición. Estas adaptaciones modifican la composición corporal, el comportamiento de ingestión y los niveles de apetito / saciedad en la progenie y contribuyen al desarrollo de lo que se conoce como "fenotipo ahorrador", descrito por primera vez en los años 1990s (Hales y Barker, 1992). Posteriormente, se ha establecido que, además de la variación genética estructural, las modificaciones epigenéticas, principalmente de metilación del ADN (Stöger, 2008), también determinan las adaptaciones de la progenie, lo que coincide con la teoría del "epigenotipo ahorrador" (Stöger, 2008). El cerdo ibérico, ha sido objeto de estudio del proceso de programación prenatal, observándose importantes diferencias en la progenie de hembras alimentadas normalmente y bajo un déficit nutricional (Gonzalez-Bulnes et al., 2012a; Barbero et al., 2014; Ovilo et al., 2014c).

1.4.3.5.2- Alimentación postnatal

Es la herramienta más frecuentemente empleada y mejor conocida para incidir en el contenido de GIM. Existen variadas estrategias para modificar la cantidad y la composición de la grasa corporal mediante la modificación de la dieta durante la vida productiva del animal:

1.4.3.6.2.1- Contenido energético de la dieta

Es un hecho ampliamente aceptado que el aumento del contenido energético de la dieta (generalmente mediante la adición de fuentes de grasa), aumenta el engrasamiento de la canal en el cerdo (Seerley et al., 1978; Coffey et al., 1982; Ellis et al., 1996; Suarez-Belloch et al., 2013). Sin embargo otros autores no han encontrado ningún efecto (De la Llata et al., 2001; Weber et al., 2006), posiblemente debido a las diferencias entre genotipos, periodos de suplementación y niveles de inclusión de grasa (Suarez-Belloch et al., 2013). En cuanto al contenido en GIM de la carne de cerdo, se han encontrado resultados diversos; algunos autores han observado un ligero efecto positivo de la adición de grasa en el pienso sobre el contenido en GIM (Cromwell et al., 1978; Liu et al., 2007), mientras que la mayoría coincide en que el nivel de energía en la dieta no tiene efecto significativo sobre la cantidad de GIM (Apple et al., 2004; Hinson et al., 2011; Alonso et al., 2012; Suarez-Belloch et al., 2013). Por todo ello, podemos concluir que la adición de grasa en la dieta tiene un mínimo reflejo sobre el contenido de GIM, mientras que el compartimento subcutáneo experimenta un incremento sustancial en contenido graso.

1.4.3.6.2.2- Contenido proteico y relación proteína / energía

La restricción proteica, así como la restricción en lisina, el primer aminoácido limitante en porcino, ha demostrado tener un mayor impacto sobre el contenido en GIM (Castell et al., 1994; Goerl et al., 1995), aumentándolo según disminuye la cantidad de proteína incorporada (Castell et al., 1994). Sin embargo, muchos de los trabajos que se mostraron efectivos incrementando la GIM, tuvieron un efecto negativo sobre el crecimiento, conversión del alimento, y contenido magro de la canal (mayor espesor de tocino dorsal y menor área de *Longissimus dorsi* (LD)) (Goerl et al., 1995; Kerr y Easter, 1995). Los trabajos más recientes han aplicado restricciones proteicas más breves en el tiempo, buscando mantener resultados adecuados de crecimiento y de calidad de la canal (Witte et al., 2000; Katsumata, 2011; Bessa et al., 2013).

Se ha observado también un aumento en el contenido en GIM en cerdos alimentados con niveles altos de leucina (Cisneros et al., 1996a; Hyun et al., 2003). La leucina es un aminoácido relevante en la síntesis proteica, que también favorece la liberación de insulina e inhibe la degradación de las proteínas. Al tratarse de un aminoácido quetogénico, su degradación da lugar a acetyl-CoA, utilizado en la síntesis de ácidos grasos en el músculo (Hyun et al., 2007).

1.4.3.6.2.3- Manipulación de la composición de ácidos grasos

Algunos estudios han demostrado que la composición de ácidos grasos no se ve afectada por el nivel energético de la dieta (Alonso et al., 2012; Suarez-Belloch et al., 2013), pero si por la fuente de grasa (Weber et al., 2006; Alonso et al., 2012) que determina la concentración de AGMI y AGPI, siendo la concentración de AGS más estable entre distintas fuentes de grasa.

Esto es debido a que gran parte de la acumulación de grasa en el cerdo se produce por acumulación directa de los ácidos grasos de la dieta. Así, se han evaluado distintas estrategias que modifican la proporción de ácidos grasos en los tejidos del cerdo para adaptarse a la demanda de los consumidores. Entre las alternativas ensayadas con éxito se encuentran el enriquecimiento con C18:1 (hasta el 60% de la grasa) y con ácidos grasos de la familia n-3, o la reducción en la concentración de AGS (hasta el 22-25%), etc. (St John et al., 1987; Miller et al., 1990; Kouba y Mourot, 2011). Por otro lado, se ha observado en pollos que dietas con un perfil de ácidos grasos más saturado aumenta el engrasamiento de la canal e incluso el contenido en GIM (Sanz et al., 2000), pero la información es poco consistente. En ganado porcino los trabajos en relación con el tema son escasos. Madsen y colaboradores (1992) observaron en cerdos que según se incrementaba el grado de saturación de las grasas suministradas a los animales se producía un incremento del contenido de GIM, a la vez que esta última disminuía según se incrementaba el contenido en grasa (tanto aceite de palma como grasa de origen animal) en el pienso. En un estudio más reciente elaborado por Flachowsky y colaboradores (2008), se incluyó un 2.5% de grasa de diferente origen a las dietas: sebo, aceite de oliva, aceite de soja y aceite de lino. En dicho experimento, no se observaron efectos significativos en relación al contenido en GIM en función del tipo de grasa utilizado. No obstante, el valor más elevado de GIM correspondió a la ración suplementada con grasa animal y el más bajo a la dieta que incluía aceite de soja.

1.4.3.6.2.4- Contenido en micronutrientes

La investigación respecto al efecto de los niveles de micronutrientes en la ración sobre el contenido en GIM es un frente de investigación abierto recientemente. Algunos de los micronutrientes de interés son el ácido linoleico conjugado (CLA) o ciertas vitaminas, entre las que destaca la vitamina A (VA).

La suplementación con CLA disminuye el espesor de la grasa subcutánea (Wiegand et al., 2002; Dunshea et al., 2005), mientras que aumenta el contenido en GIM (Wiegand et al., 2002; López-Bote et al., 2008; Cordero et al., 2010). También altera el perfil de ácidos grasos, tanto en GIM como en subcutánea (Dunshea et al., 2005; Weber et al., 2006; López-Bote et al., 2008; Cordero et al., 2010). En un ensayo *in vitro* llevado a cabo por Zhou y colaboradores (2007), se usaron células del estroma vascular procedentes de tejido adiposo subcutáneo o de tejido adiposo intramuscular. La

incorporación de CLA al medio de cultivo de dichas células, supuso una disminución en la acumulación de grasa y en la expresión de genes específicos de los adipocitos en el caso de células derivadas de tejido adiposo subcutáneo y un aumento de dichos genes en células procedentes de tejido adiposo intramuscular.

En cuanto al contenido en VA, algunos autores han evaluado distintos niveles de incorporación en la dieta y no han observado ningún efecto sobre parámetros productivos, como el crecimiento, la ingesta diaria o el índice de conversión en distintas especies (D'Souza et al., 2003; Dikeman, 2007; Olivares et al., 2009a). Por otro lado, otros estudios han demostrado que la VA ejerce un efecto modulador sobre la adipogénesis y por lo tanto, sobre el desarrollo del tejido adiposo (Bonet et al., 2003; Olivares et al., 2011).

Existen numerosos factores que pueden influir sobre el metabolismo y la función de la VA, como son, la genética, la duración y severidad del tratamiento o la edad a la que se inicia (Olivares et al., 2009b; Olivares et al., 2011). Así, los resultados observados en distintos ensayos muestran una influencia variable de la VA sobre el contenido en GIM, siendo por tanto necesarios nuevos estudios que determinen los mecanismos moleculares que modulan el efecto de la VA sobre la acumulación de grasa.

1.5- ESTRATEGIAS ESTUDIADAS PARA MODIFICAR LA GRASA INTRAMUSCULAR EN EL CERDO IBÉRICO

1.5.1- Efecto del tipo genético sobre la grasa intramuscular

1.5.1.1- El cruce ibérico-Duroc

El cerdo ibérico ha sido tradicionalmente cruzado con líneas modernas para mejorar los rendimientos productivos, siempre intentando mantener las características de la raza de relevancia para el consumidor, como la capa oscura y el color negro de la pezuña. Es por ello que se ha cruzado con otras razas que comparten estas características, como Large Black, Berkshire o Duroc. Esta última raza ha sido la más frecuentemente utilizada para mejorar la prolificidad, la tasa y eficiencia de crecimiento y el contenido magro sin afectar gravemente a los parámetros de calidad de los productos (López-Bote, 1998).

En la actualidad, el cruce de cerdo ibérico está aceptado exclusivamente con cerdos Duroc. Como se ha mencionado anteriormente, una gran proporción del total de cerdos sacrificados bajo la Norma de calidad de los productos del ibérico (Real Decreto 4/2014) corresponde a animales ibéricos cruzados al 50% con Duroc. Para asegurar el mantenimiento de la variabilidad genética y de la población de raza ibérica, la genética Duroc sólo puede ser utilizada como línea paterna terminal.

Sin embargo, se ha descrito un impacto negativo de la introducción de genética Duroc sobre la calidad de los productos ibéricos en diversas publicaciones científicas. Algunos de los factores que pueden ser alterados por el empleo de genética Duroc son:

- La disminución de la GIM y de los AGMI en los animales cruzados (Ventanas et al., 2006; Fuentes et al., 2014). Es el efecto más importante desde el punto de vista de la calidad de los productos ibéricos.
- Las cualidades sensoriales: los jamones ibéricos fueron considerados ligeramente más amargos (Carrapiso et al., 2003) y los lomos curados más jugosos (Ventanas et al., 2007) que los procedentes de animales cruzados.
- El veteado en jamones y lomos curados (Carrapiso et al., 2003; Ventanas et al., 2007).
- El color: los jamones ibéricos mostraron mayor predominio del color rojo en fresco, aunque esta diferencia no se mantuvo tras el curado (Andrés et al., 2001). El brillo también fue mayor en lomos curados de cerdos ibéricos puros (Andrés et al., 2001; Ventanas et al., 2007).
- El perfil de los triglicéridos presentes en la grasa (Tejeda et al., 2002; Petrón et al., 2004).

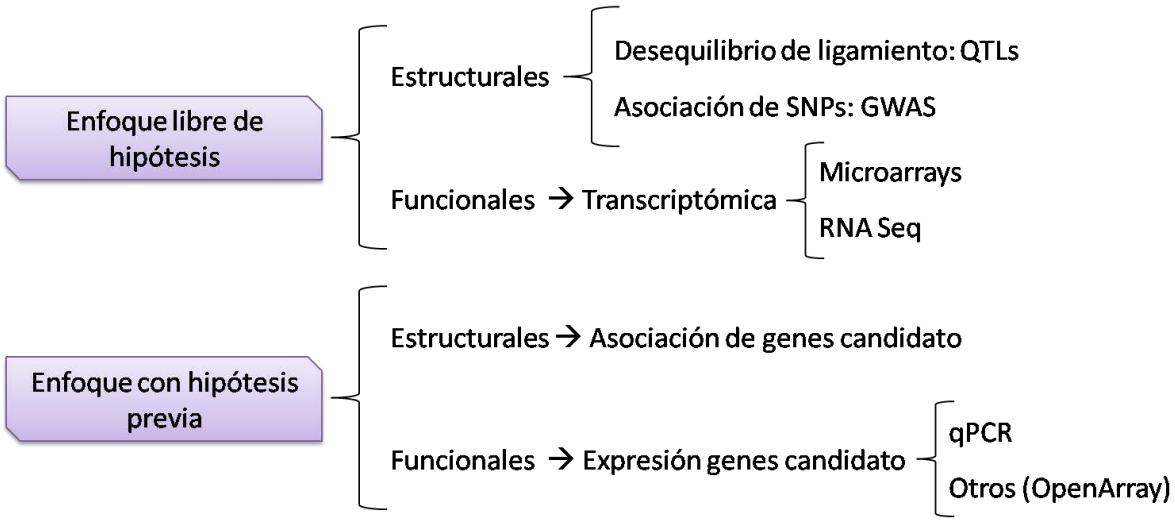
Todos estos cambios en la composición y en las características del músculo y de la grasa están determinados por factores genéticos, como la mutación descrita por Van Laere y colaboradores

(2003) en el gen *IGF-2*, un claro gen candidato relacionado con el crecimiento muscular y con la calidad de la carne. Sin embargo, el conocimiento sobre los mecanismos moleculares implicados en las diferencias en calidad de carne entre cerdos ibéricos puros y cruzados con Duroc es todavía limitado.

1.5.1.2- La metodología molecular al servicio de la mejora genética animal

Una gran variedad de herramientas moleculares y métodos estadísticos han sido empleados en el área de la mejora genética animal con el fin de conocer la base genética de la regulación de caracteres complejos como son el crecimiento, la acumulación de grasa, la prolificidad, etc. La figura 14 muestra, de modo resumido, los distintos tipos de enfoques utilizados en estudios genéticos. Podemos diferenciar dos grandes tipos de estudios, en función de si la aproximación del trabajo es mediante el estudio de todo el genoma, sin tener en cuenta consideraciones previas (enfoque libre de hipótesis o *hypothesis-free* en inglés) o si es el estudio de un número limitado de genes (genes candidato), que han sido previamente seleccionados por estar potencialmente relacionados con el fenotipo (enfoque con hipótesis previa o *hypothesis-driven*). Este último enfoque cuenta con la debilidad de que es necesario un conocimiento biológico previo (que puede ser correcto o no) para seleccionar los genes candidato y con la fortaleza de una gran eficiencia estadística y de la comprensión biológica de las diferencias genéticas y fenotípicas observadas (Tabor et al., 2002).

Figura 14: Esquema de los tipos de estudios y metodologías utilizadas en genética molecular.

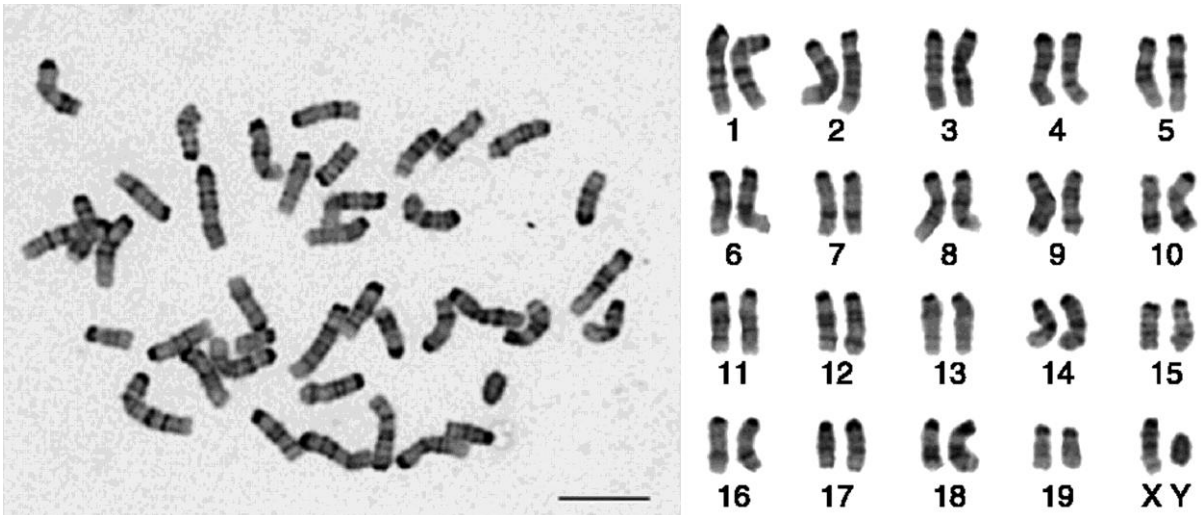


Durante los años 90 empezaron a realizarse numerosos estudios funcionales (o de expresión) y estructurales (centrados en las variaciones en la secuencia del ADN) en genes candidato. También en la misma década, numerosos estudios utilizaron marcadores de tipo microsatélite, con el fin de construir mapas genéticos y detectar regiones del genoma o *quantitative trait loci* (QTL) asociados

a determinados caracteres (Ollivier, 2009). El primer estudio de este tipo en animales domésticos fue llevado a cabo por Andersson y colaboradores (1994) en un cruce de jabalí x Large White centrado en la detección de QTLs asociados a caracteres de crecimiento y acumulación de grasa. Posteriormente, se han desarrollado un gran número de estudios para confirmar o detectar nuevos QTLs asociados a caracteres de interés como calidad de la carne, sistema inmune, reproducción o producción (www.animalgenome.org/QTLdb/pig.html). Esta estrategia presentaba ciertas limitaciones debido al uso de microsatélites para detectar los QTLs. Recientemente se han desarrollado para todas las especies domésticas plataformas de genotipado masivo de marcadores tipo SNP (*Single Nucleotide Polimorphism*), que permiten el genotipado de miles de SNPs distribuidos uniformemente a lo largo de todo el genoma (Fan et al., 2010). Entre sus aplicaciones, cabe destacar los estudios de asociación genómica, también conocidos como GWAS. En dichos estudios se relacionan datos de registros fenotípicos de caracteres de interés, con los datos de genotipado de un panel denso de marcadores representativo de todo el genoma para detectar asociaciones entre los caracteres de interés y los marcadores analizados (Goddard y Hayes, 2009).

Sin duda, la secuenciación del genoma porcino, iniciada en el año 2003 ha contribuido enormemente a la detección de nuevas regiones genómicas asociadas a caracteres de interés. El genoma porcino está compuesto por 18 pares de autosomas y dos cromosomas sexuales (Figura 15) y con un tamaño estimado de alrededor de 2.7 Gb (Walters et al., 2012). En primer lugar se llevó a cabo la secuenciación de una hembra de la raza Duroc usando cromosomas artificiales de bacterias (BACs) obteniendo una cobertura de 4x (Archibald et al., 2010). Desde entonces, se han puesto a disposición de la comunidad investigadora diversas versiones del genoma porcino que se han revisado con el objetivo de mejorarlas al máximo hasta la actual versión Sscrofa 10.2 (Groenen et al., 2012).

Figura 15: Cariotipo normal del cerdo. (Fuente: Watanabe et al., 2010)

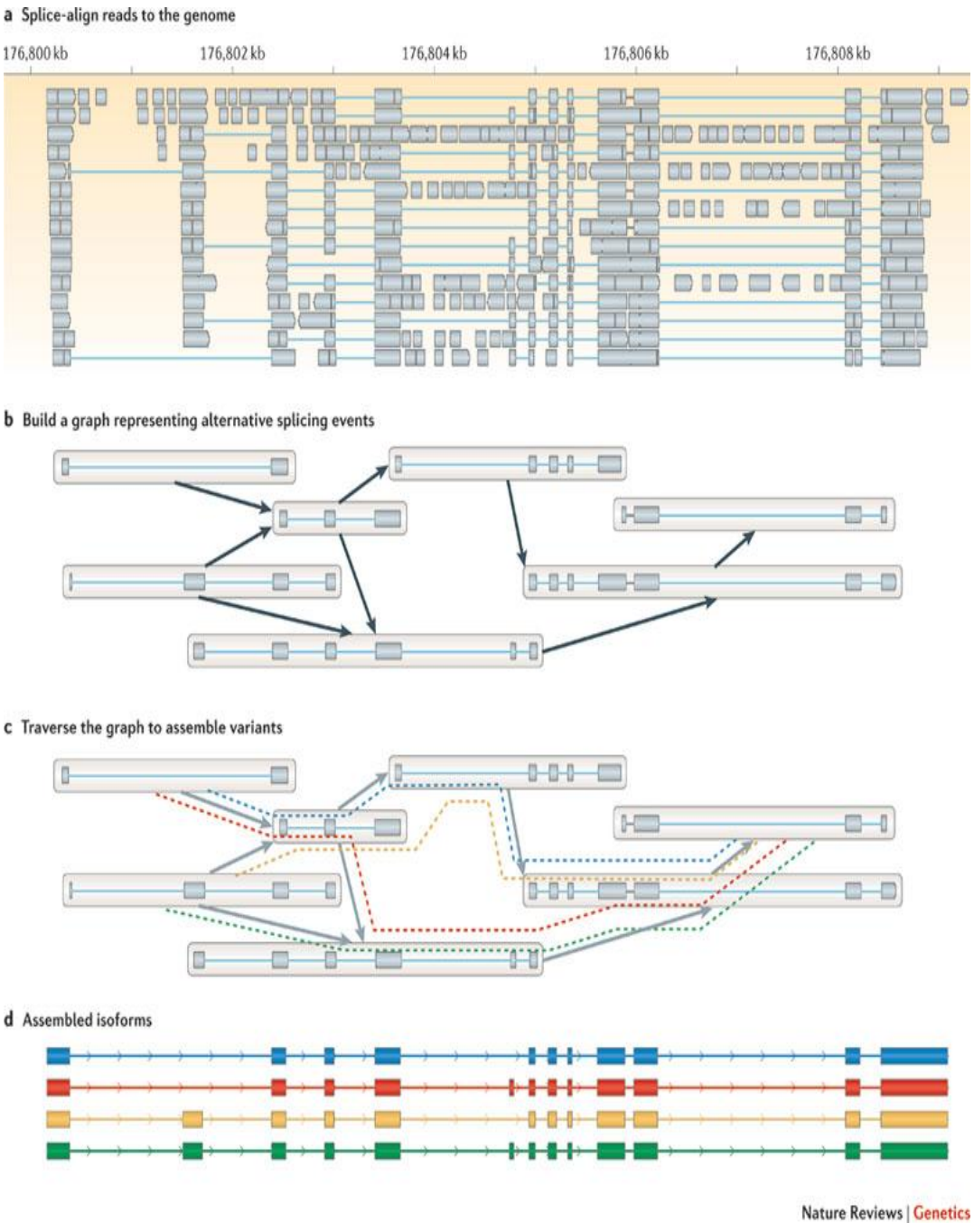


La secuenciación del genoma porcino ha permitido también el avance en el desarrollo y aplicación de las tecnologías de análisis del transcriptoma en esta especie. En primer lugar se desarrollaron los microarrays, que permitían analizar la expresión de miles de genes conocidos. En concreto para la especie porcina se han desarrollado dos chips comerciales, el chip de la plataforma *Affymetrix Porcine Gene Chip™*, que contiene 23,937 conjuntos de sondas correspondientes a 23,256 tránscritos de 20,201 genes conocidos y el *Agilent Porcine Gene Expression Microarray* que contiene 43,803 sondas. Sin embargo, en los últimos años la rápida evolución de las tecnologías de secuenciación masiva han revolucionado las técnicas de análisis global de la expresión génica. La secuenciación masiva del transcriptoma realizada mediante la técnica conocida como RNA-seq permite analizar la gran complejidad del transcriptoma generando una visión global sin precedentes permitiendo un análisis mucho más exhaustivo (Chen et al., 2011). Esta estrategia presenta varias ventajas frente al uso de microarrays. En primer lugar, tiene mayor sensibilidad y rango dinámico y menor variación técnica y ruido, además requiere menor cantidad de ARN de partida (Oshlack et al., 2010; Chen et al., 2011). En segundo lugar, mediante RNA-seq es posible capturar casi todos los tránscritos expresados, mientras que los análisis basados en microarrays dependen de información a priori y no son capaces de detectar nuevos genes o tránscritos. Permite también la cuantificación de la expresión de cada tránscrito, de ARNs no codificantes y de mutaciones post transcripcionales (Wang et al., 2009). Por último, es posible investigar no sólo cambios en la expresión génica, sino también cambios estructurales en el ARNm como SNPs y otras variantes estructurales, lo que permite a su vez determinar la expresión génica de forma alelo-específica (Qian et al., 2014).

El procedimiento general (Figura 16) en este tipo de análisis consiste en la fragmentación del ARN y secuenciación de fragmentos cortos mediante alguna de las tecnologías disponibles en el mercado como la de Illumina, SOLiD o Roche, generando secuencias de entre 35 y 500pb (Martin y Wang, 2011). Posteriormente estas lecturas o secuencias cortas son mapeadas frente al genoma de referencia, en el caso de estar disponible, o alineadas *de novo*. En un tercer paso, las lecturas son ensambladas en fragmentos más largos dentro de los genes o tránscritos. El análisis del nivel de expresión de cada tránscrito puede realizarse debido a que el número de lecturas obtenidas es proporcional al nivel de expresión. Así, una vez normalizados los datos es posible obtener los niveles de expresión de cada tránscrito y realizar análisis de expresión diferencial (Oshlack et al., 2010).

El objetivo de un estudio de expresión diferencial consiste en identificar los genes cuya expresión ha cambiado significativamente entre dos condiciones diferentes. Es posible identificar no solo genes diferencialmente expresados (DE) sino también isoformas DE, diferente uso de promotores y diferentes sitios de inicio de la transcripción (Trapnell et al., 2012).

Figura 16: Representación del proceso general de la construcción de bibliotecas (a) y de ensamblado del transcriptoma basado en un genoma de referencia (b). (Fuente: Martin y Wang, 2011).



A pesar de las grandes ventajas que presenta esta tecnología, es necesario tener en cuenta que también presenta algunas limitaciones, desde fallos en el procesamiento y secuenciación de las muestras hasta el análisis bioinformático de los datos. La mayoría de los sesgos en la construcción de las librerías y en la secuenciación quedan solventados con el uso de lecturas pareadas. Sin

embargo, es necesario seguir implementando los métodos de análisis de los datos obtenidos, además de homogeneizar el protocolo de análisis, lo que resulta complicado teniendo en cuenta los variados softwares y estrategias de análisis disponibles. Por otro lado, la cantidad de información que generan este tipo de experimentos (del orden de 5 GB por archivo), encarece y dificulta el almacenamiento (Mantione et al., 2014) (Chen et al., 2011).

Estas dos últimas metodologías (microarrays y RNA-Seq) se han aplicado de forma extensa en la especie porcina con el fin de comprender los aspectos genéticos asociados a caracteres fenotípicos de interés. Numerosos estudios han investigado las bases genéticas relacionadas con el desarrollo muscular (Cagnazzo et al., 2006; Kim et al., 2010; D'Andrea et al., 2011; Zhao et al., 2011; Damon et al., 2012; Guo et al., 2014; Ovilo et al., 2014b), que históricamente han despertado un gran interés en los científicos debido a la importancia económica que supone en la producción porcina y en la de otras especies ganaderas, así como con la resistencia a enfermedades y desarrollo del sistema inmune (Bao et al., 2012; Mach et al., 2013; Xing et al., 2014). También se ha estudiado el transcriptoma en relación a caracteres reproductivos (Samborski et al., 2013) y a factores relacionados con la calidad de la carne, como pueden ser la composición de ácidos grasos (Puig-Oliveras et al., 2014) o el engrasamiento (Pérez-Montarelo et al., 2014; Xing et al., 2015). La mayoría de estos estudios se ha realizado en razas comerciales (Sodhi et al., 2014) y en una gran variedad de tejidos. Los trabajos cuyo objetivo fue determinar genes implicados en el crecimiento o desarrollo muscular o en la calidad de la carne investigaron preferentemente el transcriptoma muscular, aunque otros tejidos, por ejemplo la grasa dorsal (Corominas et al., 2013a) o tejidos con función endocrina como la glándula tiroides, las gónadas (Pérez-Enciso et al., 2009) o incluso el hipotálamo (Pérez-Enciso et al., 2009; Pérez-Montarelo et al., 2014) han sido también objeto de estudio. Por otro lado, se ha investigado el transcriptoma de tejido reproductor tanto en machos como hembras (Esteve-Codina et al., 2011; Samborski et al., 2013; Fischer et al., 2015), con el fin de identificar genes que regulen esta función. En definitiva, cualquier tejido orgánico puede ser muestreado *postmortem* para analizar su transcriptoma y otros como la sangre o el tejido adiposo permiten el muestreo secuencial in vivo. La elección de uno u otro dependerá del objeto del estudio. La aplicación de estas técnicas en razas tradicionales es más modesta y se basa sobre todo en la comparación del transcriptoma de este tipo de razas con el de razas modernas, habitualmente muy divergentes de las primeras. Algunos de estos estudios, como los llevados a cabo en la raza china Jeju (Sodhi et al., 2014; Ghosh et al., 2015) o en la italiana Casertana (D'Andrea et al., 2011) encontraron diferencias en la expresión génica asociadas a parámetros tan interesantes desde el punto de vista productivo como el crecimiento, el desarrollo del músculo esquelético, el metabolismo lipídico o la respuesta inmune. En el caso del cerdo ibérico, los trabajos realizados en este ámbito son escasos (Tabla 4).

Tabla 4: Estudios del transcriptoma realizados en animales de raza ibérica

Metodología	Objetivo	Tejido	Raza/s	Referencia
Microarray	Expresión diferencial en animales extremos para composición de AG	Músculo	IB x LD	(Pena et al., 2013)
RNA Seq	Comparación entre razas	Testículo	IB y LW	(Esteve-Codina et al., 2011)
Microarray	Modelo animal de respuesta inmune	Neutrófilos	IB	(Sanz-Santos et al., 2011)
Microarray	Expresión diferencial en animales extremos para prolificidad	Ovario	IB x MS	(Fernandez-Rodriguez et al., 2011)
Microarray	Comparación entre razas y tejidos	16 Tejidos	IB y LW	(Ferraz et al., 2008)
RNA Seq	Expresión diferencial en animales extremos para composición de AG	Músculo	IB x LD	(Puig-Oliveras et al., 2014)
RNA Seq	Expresión diferencial en animales extremos para crecimiento y engrasamiento	Hipotálamo	IB x LD	(Perez-Montarelo et al., 2014)
RNA Seq	Expresión diferencial en animales extremos para composición de AG de la GIM	Grasa	IB x LD	(Corominas et al., 2013a)
Microarray	Efecto de la fuente de energía de la dieta sobre la expresión génica	Grasa	IB	(Ovilo et al., 2014a)
RNA Seq	Expresión diferencial en animales extremos para composición de AG de la GIM	Hígado	IB x LD	(Ramayo-Caldas et al., 2012)
Microarray	Comparación entre razas y tejidos	5 Tejidos endocrinos	DU, LW, IB, híbrido	(Perez-Enciso et al., 2009)
Microarray	Comparación entre genotipos	Músculo	IB, IB x DU	(Ovilo et al., 2014b)

IB: Ibérico
LW: Large White
MS: Meishan
AG: Ácidos grasos
GIM: Grasa intramuscular
DU: Duroc

Un buen número de estudios en los que se ha analizado el transcriptoma del cerdo ibérico puro o cruzado, han estado encaminados a encontrar genes DE en animales con fenotipos divergentes para ciertos caracteres relacionados con parámetros productivos o de calidad de carne, como el crecimiento o la cantidad y la composición de la GIM o la grasa subcutánea. Para ello, se ha cruzado el cerdo ibérico con razas muy diferentes desde un punto de vista fenotípico y genotípico, lo que facilita la obtención de animales con una gran variabilidad. Los animales fueron fenotipados y, tras un análisis de componentes principales (PCA), se identificaron los animales con fenotipos extremos

para los caracteres de interés. El análisis del transcriptoma se realizó sobre estos individuos en distintos tejidos (hígado, músculo, hipotálamo o grasa). Estos estudios (Ramayo-Caldas et al., 2012; Corominas et al., 2013a; Perez-Montarelo et al., 2014; Puig-Oliveras et al., 2014) permitieron identificar genes DE y rutas metabólicas enriquecidas entre cerdos de fenotipos extremos relacionados con el crecimiento, el engrasamiento o la composición de la grasa. La información generada sobre los mecanismos genéticos involucrados en estas diferencias fenotípicas proporciona el conocimiento base sobre el cual se puedan diseñar futuros estudios que permitan identificar mutaciones causales en alguno de los genes candidato propuesto previamente, lo que repercutiría económicamente en el sector porcino. Sin embargo, también puede ser relevante desde el punto de vista de la salud humana, puesto que muchos de estos mecanismos relacionados con el crecimiento y el metabolismo y acumulación de la grasa pueden ser comunes entre ambas especies, lo que contribuiría a la investigación de enfermedades como la obesidad, el síndrome metabólico o la diabetes tipo 2. El cerdo ibérico es una raza especialmente interesante como modelo biomédico de investigación de procesos de obesidad, dado su peculiar metabolismo y propensión al engrasamiento (Torres-Rovira et al., 2012; Barbero et al., 2014; Ovilo et al., 2014c). Además, se ha realizado otro estudio en cerdo ibérico empleando este animal como modelo animal de la respuesta inmune innata frente a lipopolisacáridos bacterianos (Sanz-Santos et al., 2011).

Por otro lado, los trabajos más antiguos que analizan el transcriptoma del cerdo ibérico se llevaron a cabo en los años 2008-2009, utilizando la técnica del microarray (Ferraz et al., 2008; Pérez-Enciso et al., 2009). Estos estudios aportaron información valiosa sobre los cambios en la expresión génica observados entre distintas razas (entre ellas, la raza ibérica) y en distintos tejidos. Desde entonces, se han publicado escasos estudios comparativos del transcriptoma del cerdo ibérico con otras razas. Por ejemplo, Esteve-Codina y colaboradores compararon en 2011 el transcriptoma testicular entre el cerdo ibérico y el Large White mediante la tecnología RNA-Seq. Estos autores observaron expresión diferencial en genes estrechamente relacionados con la espermatogénesis y el metabolismo lipídico, de acuerdo con las diferencias fenotípicas en cuanto a prolificidad y acumulación de grasa observadas entre ambas razas. Posteriormente, en el año 2014, Óvilo y colaboradores realizaron una comparación del transcriptoma del músculo LD del cerdo ibérico con un tipo genético mucho más cercano que el empleado por Esteve-Codina y colaboradores (2011), un cruce de ibérico con Duroc (50%). Estos dos tipos genéticos son de gran importancia para el sector del porcino ibérico, puesto que son los dos tipos genéticos aceptados por el Real Decreto 4/2014 para la obtención y elaboración de productos etiquetados como “ibérico”. Pese a la cercanía de los genotipos comparados y a la temprana edad a la que se realizó el estudio (28 días), los autores encontraron diferencias marcadas, tanto a nivel fenotípico (los animales puros presentaron mayor GIM que los cruzados) como a nivel de expresión, detectando 250 genes DE que además se relacionaron con procesos tan interesantes desde el punto de vista del desarrollo muscular y la

acumulación de grasa como el desarrollo de la matriz extracelular, la proteólisis o el metabolismo lipídico. En este trabajo se identificaron además potenciales genes reguladores responsables de las diferencias en expresión génica y por lo tanto en características fenotípicas entre ambos tipos genéticos. En este trabajo se utilizó la tecnología de microarray que, como se ha señalado previamente, presenta desventajas frente a la secuenciación masiva del ARN (RNA-Seq). Por ello, es de gran interés la aplicación de esta nueva tecnología para seguir investigando las diferencias en la transcripción entre estos dos genotipos aprovechando por ejemplo la mayor sensibilidad de la técnica o la capacidad de estudiar el transcriptoma a nivel estructural.

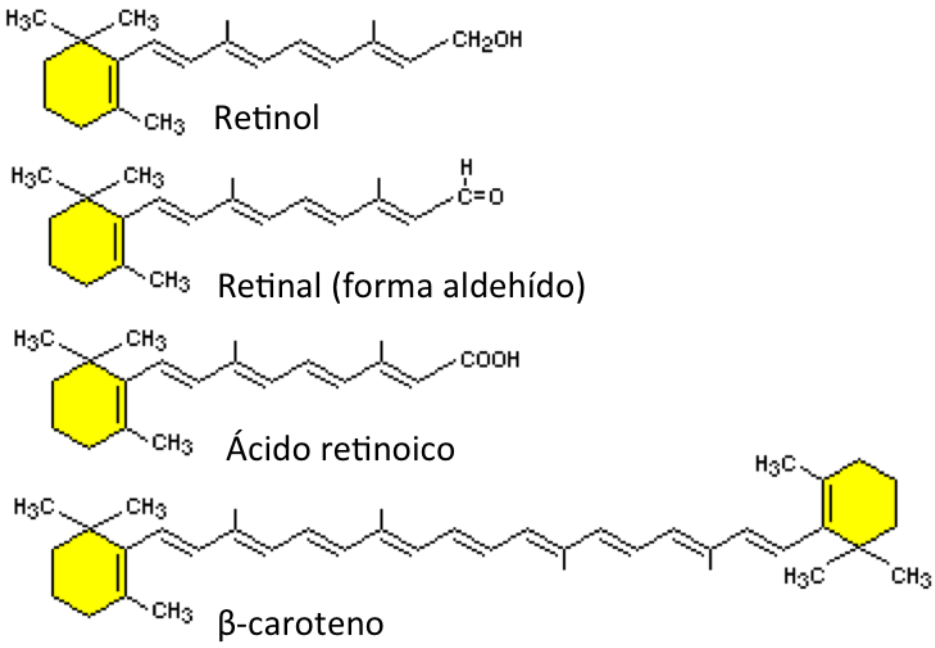
1.5.2- Efecto de la vitamina a sobre la grasa intramuscular

La VA, es un alcohol poliénico isoprenoide de 20 átomos de carbono. Se conoce también con otros nombres como retinol, axeroftol, biosterol, vitamina antixeroftálmica y vitamina antiinfecciosa.

1.5.2.1- Estructura Química

El retinol puede presentarse en forma esterificada constituyendo los ésteres *all-trans retinol*. Mayoritariamente la esterificación tiene lugar con el ácido palmítico (aunque también puede producirse con el acético y el propiónico) formándose así el palmitato de retinol. Si el grupo terminal del retinol es un grupo carboxilo, se conoce al compuesto como ácido retinoico (Figura 16). La forma aldehído del retinol, de elevada importancia por el papel que juega en la visión, se conoce como *11- cis retinal* (Figura 17). La unidad internacional de VA equivale a 0,3 µg de retinol (1 mg retinol = 3333 UI de VA), 0,344 µg de acetato de retinol, 0,359 µg de propionato de retinol ó 0,55 µg de palmitato de retinol. En general, todas las formas de presentación de la VA son denominados retinoides.

Figura 17: Estructura química de las distintas formas de presentación de la vitamina A.
(Fuente: http://www.vivo.colostate.edu/hbooks/pathphys/misc_topics/vitamina.html)



En los alimentos de origen animal, la VA se presenta, en su mayor proporción, como retinol esterificado con el ácido palmítico. En los vegetales y en algunos organismos marinos, la encontramos en forma de provitamina, como ciertos carotenoides. Se conocen cerca de 100 moléculas con actividad provitamina A. Entre ellas el β- caroteno es el más abundante y activo; se trata de un pigmento amarillo constituido por dos moléculas de retinal unidas en el extremo aldehído de sus cadenas carbonadas (Figura 17).

1.5.2.2- Absorción y metabolismo

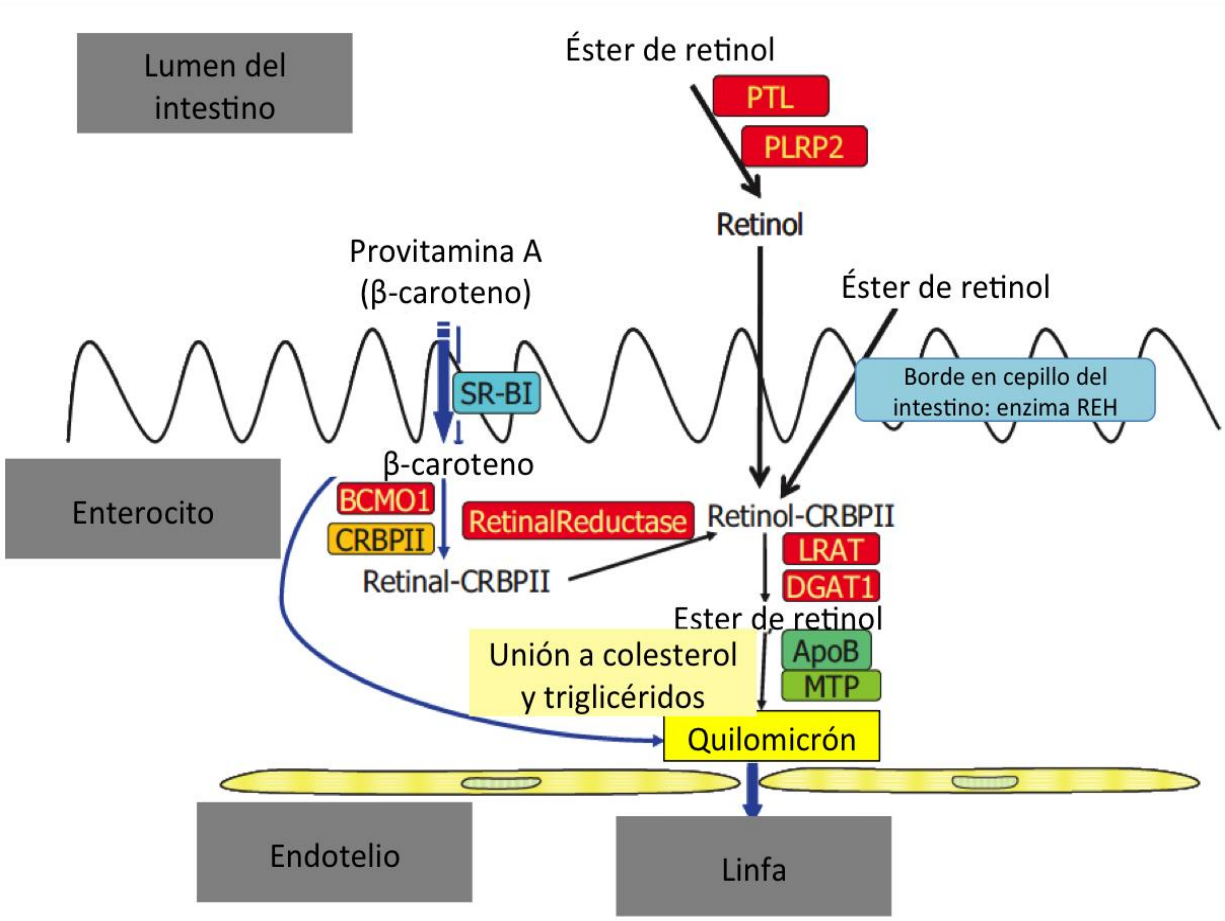
El proceso de digestión/absorción comienza después de la ingestión del alimento, cuando el β-caroteno o provitamina A es liberado de las proteínas a las que se encuentra unido por la acción de la pepsina en el estómago y de otras enzimas proteolíticas (Ross, 1993). A continuación, la provitamina A entra al interior del enterocito mediante un proceso que requiere de la intervención del receptor de membrana SR-B1 (scavenger receptor class B, type I) (Figura 18). Las formas esterificadas del retinol deben hidrolizarse mediante la acción de las enzimas PTL y PRLP2 para liberar el alcohol (retinol), que es la molécula que realmente se absorbe. La formación de micelas facilita la digestión y la acción enzimática al aumentar la superficie de interfase agua-lípido. Para ello, es necesario el consumo de grasas junto con los retinoides (D'Ambrosio et al., 2011). El paso de la forma éster a alcohol también se produce en el borde en cepillo de los enterocitos (Figura 18),

por acción de la enzima retinol esterhidrolasa (REH) (D'Ambrosio et al., 2011). La eficiencia de absorción del retinol en el intestino de la rata se estima alrededor del 80-90 %, mientras que la del β-caroteno es del 60-70% (Olson, 1961).

Una vez dentro del enterocito, el retinol forma un complejo con una proteína de unión celular (CRBPII, celular retinol binding protein tipo 2) que sirve como sustrato para la reesterificación del retinol por la acción de las enzimas lecitin-retinol aciltransferasa (LRAT), que cataliza cerca del 90% de la reesterificación y diacilglicerol aciltransferasa 1 (DGAT1), que se encarga de catalizar el resto. El β -caroteno, por su parte puede seguir dos rutas diferentes una vez ha penetrado en el enterocito; en primer lugar, puede ser metabolizado a la forma aldehído mediante la enzima BCMO1 (Beta caroteno monoxigenasa 1) y a continuación ser reducido a retinol (Figura 18); por otra parte, el β -caroteno puede también ser incorporado directamente en los quilomicrones.

Los ésteres de retinol se incorporan posteriormente al interior de los quilomicrones linfáticos, lipoproteínas intestinales que contienen otros lípidos tales como triglicéridos, fosfolípidos, colesterol, ésteres de colesterol y apolipoproteina B. La incorporación de algunos de estos lípidos es dependiente de la actividad de la proteína de transferencia de triglicéridos microsomales (MTP) (Harrison, 2005). Los quilomicrones con los ésteres de retinol en su interior son secretados a la linfa y a la circulación portal, llegando de esta manera hasta el hígado (D'Ambrosio et al., 2011). Al convertirse en quilomicrones remanentes, el hígado los capta para incorporar con ellos los ésteres retinol que contienen. De esta manera el retinol se almacena en dicho órgano, que se estima que contiene alrededor del 90% de la VA del cuerpo (Groff et al., 1995). Las células estrelladas del hígado juegan un papel central en la acumulación de retinoides en este órgano. Para mantener los niveles plasmáticos adecuados de VA, el hígado hidroliza los depósitos de ésteres a retinol libre, que es liberado al torrente sanguíneo unido a proteínas plasmáticas transportadoras de retinol (RBP). Estas proteínas son en su mayoría sintetizadas y secretadas por las células parenquimatosas hepáticas, aunque una pequeña cantidad se sintetiza en otros órganos del animal como el riñón o el endometrio uterino (Clawitter et al., 1990). Una vez que el retinol llega a las células diana, la presencia de la proteína transmembrana estimulada por AR (STRA6), facilita su entrada a la célula, donde es metabolizado a la forma aldehído (retinal), mediante una reacción reversible catabolizada por enzimas aldehído deshidrogenasas (ADHs) (Pares et al., 2008). A continuación se produce la oxidación irreversible del retinal a AR mediante la enzima RALDH (retinaldehído deshidrogenasa) (Duester et al., 2003).

Figura 18: Esquema de los procesos de absorción y metabolismo de la vitamina A en el enterocito. (Fuente: Adaptado de D'Ambrosio et al., 2011)



1.5.2.4- Interacciones con la vitamina E

El término vitamina E engloba una serie de compuestos antioxidantes fenólicos liposolubles constituidos por cuatro isómeros, α, β, γ y δ, que se diferencian por el número y posición de grupos metilo unidos al anillo fenólico. Están también formados por una cadena hidrocarbonada que puede ser saturada (tocol) o insaturada (tocotrienol) (Shahidi et al., 1992). El α-tocoferol es el isómero más activo y más abundante en los tejidos animales. Está disponible comercialmente para la alimentación animal al haber sido sintetizado químicamente. La vitamina E es el principal antioxidante *in vivo*, que se localiza principalmente en el interior de las membranas celulares y previene específicamente la oxidación de los AGPI que constituyen los fosfolípidos de membrana (Halliwell, 1994). De esta forma, permite mantener la integridad estructural de las membranas, incluso en presencia de radicales libres (Diplock, 1983). Además, colabora en el mantenimiento de la fisiología celular, debido al papel que juegan los AGPI de la membrana en la generación de

compuestos metabólicamente muy activos (prostaglandinas, prostaciclinas, tromboxanos, leucotrienos). Los efectos de esta vitamina se extienden a los sistemas reproductor, muscular, circulatorio, nervioso, e inmune. Dentro del ámbito de la producción de carne, numerosos estudios han demostrado la existencia de una relación positiva entre la administración de cantidades elevadas de vitamina E y algunos atributos de calidad de la carne. Entre estos atributos cabe destacar que la vitamina E mejora la estabilidad oxidativa de la carne (Rey et al., 1997; Daza et al., 2005; Rey et al., 2006a), la estabilidad del color (Monahan et al., 1994) y reduce las pérdidas de exudado (Asghar et al., 1991; Monahan et al., 1994). Por todo ello, es importante aportar un suplementación suficiente de vitamina E, en especial en etapas tardías de la producción de las distintas especies ganaderas, incluyendo el cerdo.

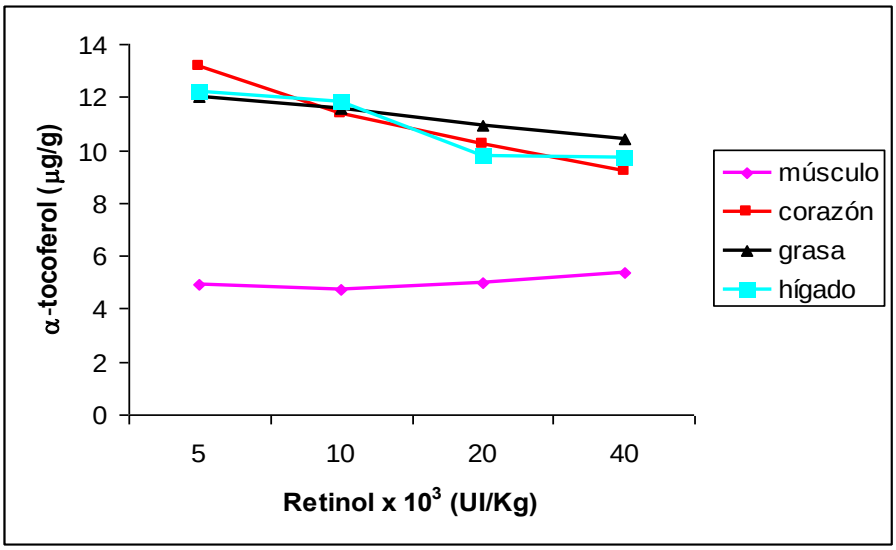
La suplementación vitamínica es una práctica habitual en alimentación animal. En el cerdo, los niveles de inclusión recomendados en España, recogidos en las normas de la Fundación Española para el Desarrollo de la Nutrición Animal (FEDNA) para la formulación de piensos (De Blas et al., 2013), superan en gran medida las necesidades vitamínicas mínimas estimadas (NRC, 2012). El exceso de suplementación puede conllevar efectos negativos, principalmente debidos a interacciones entre nutrientes, de modo que la presencia en exceso de uno de ellos limita la biodisponibilidad del otro. La interacción entre las vitaminas A y E ha sido descrita en numerosas especies como ratas (Bieri et al., 1981; Sklan y Donoghue, 1982; Abawi y Sullivan, 1989; Blakely et al., 1991; Eicher et al., 1997) y seres humanos (Goodman et al., 1994; de Lira et al., 2013).

En ganado porcino, los resultados bibliográficos son consistentes en lechones jóvenes. Ching y colaboradores (2002) observaron en lechones destetados que los animales que habían recibido una mayor suplementación de acetato de retinol (13,200 frente a 2,200 IU/kg) en el pienso durante 5 semanas mostraron niveles más bajos de α-tocoferol en suero e hígado que los que habían recibido un nivel más bajo. Blair y colaboradores (1996) observaron, también en lechones, el mismo efecto en suero e hígado. Sin embargo, durante la fase de cebo, existe cierta controversia en la bibliografía existente. Así, Hoppe y colaboradores (1991), observaron que la inclusión de niveles crecientes de VA (5,000, 10,000, 20,000, 40,000 UI/kg pienso), combinado con niveles estables de vitamina E (54 UI/ kg pienso) durante 5 meses, provocaba una disminución de los niveles de α-tocoferol en el músculo cardíaco e hígado, mientras que en el músculo LD y en la grasa subcutánea no se observaron tales diferencias (Figura 19). Por el contrario, Anderson y colaboradores (1995) en un estudio de 3 meses de duración que incluía tres niveles de vitamina E (0, 15 y 150 UI/ kg pienso) y dos niveles de VA (2,000 *vs.* 20,000 UI/ kg pienso) no observaron diferencias del contenido de α-tocoferol en los distintos tejidos. Más recientemente, Olivares y colaboradores (2009c) observaron, en cerdos en crecimiento, una disminución drástica de los depósitos de α-tocoferol en grasa e hígado tras la suplementación con altos niveles de VA (100,000 UI). Sin embargo, suplementaciones más moderadas con VA (1,300 y 13,000 UI) durante 11 semanas no produjeron ningún efecto sobre los

niveles de acumulación de α-tocoferol, si bien es cierto que la retirada de dicha suplementación conllevó un aumento del α-tocoferol hepático.

Las causas por las que se produce esta interacción no se conocen por completo. La liposolubilidad es una característica común de la vitamina E y la VA que hace que compartan mecanismos de absorción, transporte y metabolismo (de Lira et al., 2013). Estas similitudes podrían ser las responsables de la interacción entre ambas vitaminas. Así, se ha sugerido que la interacción se produce durante la absorción, quizás por una alteración en la cantidad y composición de la bilis a cargo de los retinoides que afectaría a la formación de micelas, imprescindible para la absorción de compuestos liposolubles como la vitamina E (Bieri et al., 1981). Sin embargo, también se ha sugerido que la VA modifica la concentración de α tocoferol en las lipoproteínas plasmáticas, involucradas en su transporte (Ametaj et al., 2000). A pesar de la existencia en la bibliografía de trabajos que investigan el efecto de la dosis de suplementación, el tiempo o la edad del animal a la que se inicia el tratamiento, no existen estudios orientados a evaluar el efecto conjunto de dichos factores sobre la acumulación de vitaminas A y E. Además, es necesario conocer los posibles mecanismos moleculares implicados en dicha interacción.

Figura 19: Contenido en α -tocoferol en distintos tejidos en función de la cantidad de retinol suministrado en la dieta en cerdos de 105 kg de peso vivo. (Fuente: Hoppe et al., 1992)



1.5.2.5- Funciones de la vitamina A

Es un hecho bien conocido que la VA ejerce una gran influencia sobre el desarrollo y la salud en los mamíferos (Ross y Ternus, 1993). Algunas de sus funciones han sido claramente demostradas

desde su descubrimiento en 1913, mientras que su implicación en otros aspectos del metabolismo es actualmente un campo de estudio abierto (Frey y Vogel, 2011).

Dentro de las funciones principales de la VA, cabe destacar su influencia sobre:

- **La visión**: Es la función más conocida, relacionada con la formación de rodopsina, una molécula capaz de absorber la luz (Hubbard y Wald, 1952) y necesaria, por tanto para la visión en color y en ambientes con escasa luz.
- **El tejido epitelial**: La VA puede modificar la permeabilidad de las membranas celulares (Scott et al., 1982), así como la secreción de mucosa en las células epiteliales (Ahmed et al., 1990; Stephensen et al., 1993). Estos factores son determinantes para la funcionalidad de este tejido como barrera protectora frente a las agresiones externas.
- **La reproducción**: Se ha observado en numerosos estudios que la suplementación con VA tiene efectos beneficiosos en la función reproductiva de los animales (Brief y Chew, 1985; Coffey y Britt, 1993; Lindemann et al., 2008), mejorando por ejemplo el tamaño de la camada o la supervivencia embrionaria. Este efecto podría ser consecuencia de un cierto papel modulador de la VA sobre los niveles de progesterona (Darroch, 2001), y sobre las condiciones del entorno uterino (Mahan y Vallet, 1997).
- **El sistema inmune**: Los animales con deficiencia en VA muestran un incremento en la frecuencia y severidad de infecciones bacterianas, protozoarias y virales. Su efecto sobre el sistema inmune se debe al mantenimiento en óptimas condiciones de las membranas mucosas, como se ha comentado anteriormente, a su efecto sobre la producción de anticuerpos (Harmon et al., 1963), y a su acción antioxidante, entre otros.
- **El crecimiento celular y la regulación de la transcripción**: El papel de la VA en el crecimiento tisular se encuentra relacionado con un incremento de los receptores para el factor de crecimiento epidérmico y de la interleucina-1 (Shin y McGrane, 1997). Además, el AR puede interferir en el crecimiento celular alterando las comunicaciones intercelulares mediante la modificación de la permeabilidad de la membrana celular. El crecimiento celular es controlado principalmente mediante cambios a nivel de la transcripción génica, estrechamente regulada por la VA, entre muchos otros factores. Se ha establecido que la VA regula la expresión de más de 500 genes (Balmer y Blomhoff, 2002), entre los que se encuentra la hormona del crecimiento (Bedo et al., 1989). Además, la VA también está involucrada en el crecimiento óseo, puesto que participa en los procesos de diferenciación osteogénica, maduración del cartílago y mineralización ósea (Allen et al., 2002).

Además de estas funciones, recientemente ha aumentado el interés de los efectos de la VA sobre el desarrollo y metabolismo del tejido adiposo y sobre enfermedades relacionadas con este órgano, como obesidad o diabetes tipo 2 (Frey y Vogel, 2011). Debido a la importancia económica de este

tejido en la producción de cerdo, especialmente en la raza ibérica, los efectos de la vitamina sobre el tejido adiposo serán tratados con una mayor profundidad a continuación.

1.5.2.6- Vitamina A y engrasamiento

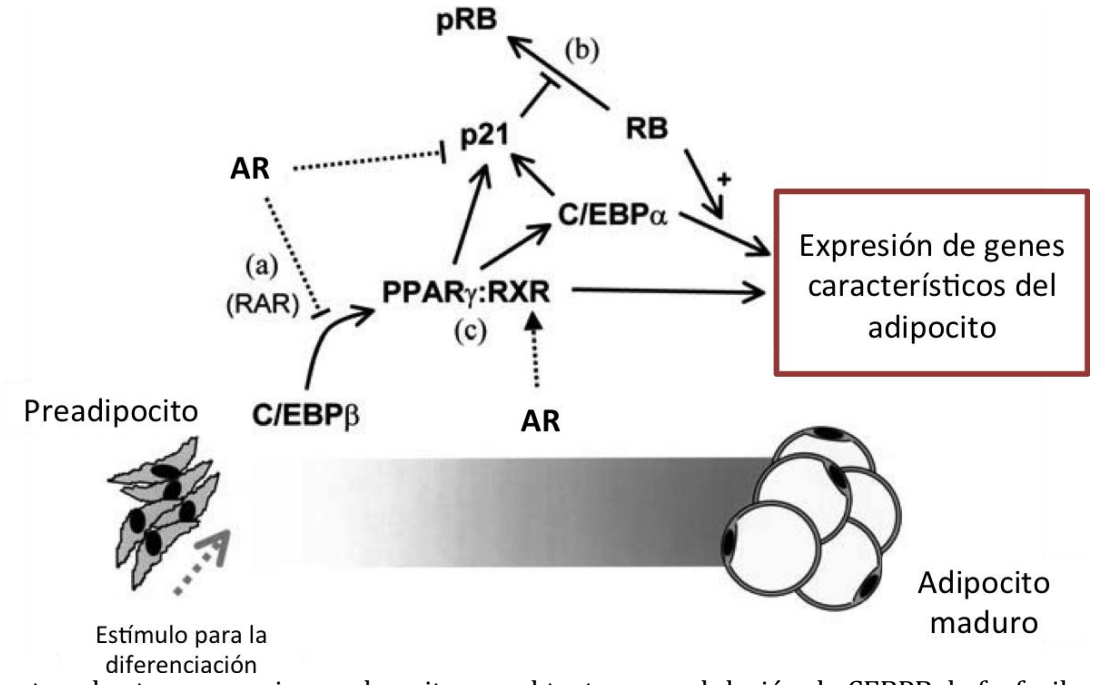
1.5.2.6.1- Efecto de la vitamina A sobre la adipogénesis.

La capacidad de modular la diferenciación adipocitaria se deriva de las propiedades de la VA como regulador de la transcripción génica y del crecimiento celular. El AR, el metabolito más activo de entre todos los retinoides, ha sido considerado un inhibidor potente de la adipogénesis desde hace 20 años (Kuri-Harcuch, 1982), cuando se observó que a altas dosis (0,1-1mM), inhibía la expresión de marcadores moleculares de la diferenciación en líneas de preadipocitos 3T3-F442A en la fase de diferenciación temprana. Además, a dosis mayores (10mM) promueve la apoptosis de preadipocitos de rata en cultivo primario (Kim et al., 2000).

La adición de AR antes o después del tratamiento con agentes inductores de la diferenciación no afecta al proceso, lo que indica que el AR actúa al inicio del proceso de diferenciación (Gregoire et al., 1998). Se han descrito distintos mecanismos por los que el AR podría ejercer sus efectos sobre este proceso:

- El AR interfiere con la actividad transcripcional de las proteínas CEBP, bloqueando la inducción de genes regulados por *CEBPB* (Schwarz et al., 1997), entre ellos *PPARG* y *CEBPA*. Para ello es necesaria la activación de los receptores RAR, principalmente *RARA* (Figura 20, a).
- Además, el AR disminuye los niveles de proteína de retinoblastoma (RB) hipofosforilada (Ribot et al., 2002) y aumenta los de RB hiperfosforilada (pRB) (Schwarz et al., 1997); esto favorece que las células mantengan su capacidad proliferativa, lo cual es incompatible con la diferenciación (Figura 20, b).
- Por último, el AR estimula la expresión de *RARG* en la línea celular 3T3-L1 mientras que disminuye la expresión de *RXRA* (Kamei et al., 1993; Kawada et al., 1996). Esto podría contribuir a la acción antiadipogénica del AR ya que favorecería la formación de dímeros RAR:RXR en lugar de PPARG:RXR (Figura 20, c).
-

Figura 20: Esquema de los efectos del ácido retinoico (AR) sobre la adipogénesis. (Fuente: Bonet et al., 2003)



Se muestran los tres mecanismos descritos en el texto; a, modulación de CEBPB; b, fosforilación de la proteína del retinoblastoma (RB/pRB, fosforilada); c, regulación de la formación de heterodímeros entre los receptores nucleares).

Sin embargo, dosis bajas de AR (1-10nM) aumentaron la adipogénesis en cultivos de células preadiposas (Safonova et al., 1994); por otro lado, se ha observado en células madre embrionarias que la incorporación del isómero *all trans-retinoic acid* favorece su compromiso hacia la línea adipocitaria (Dani et al., 1997; Bost et al., 2002). Sin embargo el mecanismo molecular a través del cual el AR ejerce su efecto promotor de la adipogénesis no se conoce (Bonet et al., 2003).

1.5.2.6.2- Efecto de la vitamina A sobre la lipogénesis.

La cantidad de tejido adiposo en el animal adulto depende del número de adipocitos y del volumen de éstos. El número de adipocitos depende de la formación de nuevas células, mediante replicación y diferenciación de precursores y de la pérdida celular por apoptosis. Ya hemos visto cómo la VA puede afectar al primer paso en el acúmulo de tejido adiposo, la diferenciación adipocitaria. Una vez que el adipocito es maduro, comenzará a almacenar gotas lipídicas en su citosol; la cantidad de lípidos almacenados depende del equilibrio entre lipogénesis y lipolisis. El AR afecta también a estos procesos, probablemente mediante su acción sobre *PPARG*, el cual destaca como un factor crítico para una correcta adipogénesis y lipogénesis (Rosen et al., 2000), ya que la mayoría de los genes diana de *PPARG* en tejido adiposo están involucrados en rutas lipogénicas. El efecto global del AR sobre el tejido adiposo es una disminución de los depósitos grasos debido a la inhibición de los procesos adipogénicos y lipogénicos y estimulación de la lipolisis y la apoptosis de células grasas (Bonet et al., 2003). Así el tratamiento con AR (100 mg *all-trans* AR/kg peso vivo) durante 4 días

produjo una fuerte disminución de los depósitos grasos en ratones (Ribot et al., 2001), mientras que una deficiencia o un aporte escaso ha sido relacionado con el efecto contrario en ratones (Ribot et al., 2001), terneros (Kawada et al., 1996) y humanos (Wolfe y Sanjur, 1988).

1.5.2.6.3- Efecto de la vitamina A sobre la desaturación de ácidos grasos.

En los mamíferos, el producto final mayoritario en la síntesis *de novo* de los ácidos grasos es el ácido palmítico (C16:0). Este ácido graso, que también puede ser de origen alimentario, se utiliza como sustrato de enzimas desaturasas y elongasas para la formación de varios AGMI y AGPI. Así, los ácidos grasos de cadena más larga se forman por reacciones de elongación catalizadas por enzimas elongasas situadas en la cara citosólica del retículo endoplasmático. Además, las enzimas desaturasas pueden introducir dobles enlaces en las cadenas hidrocarbonadas por medio de reacciones de desaturación oxidativa.

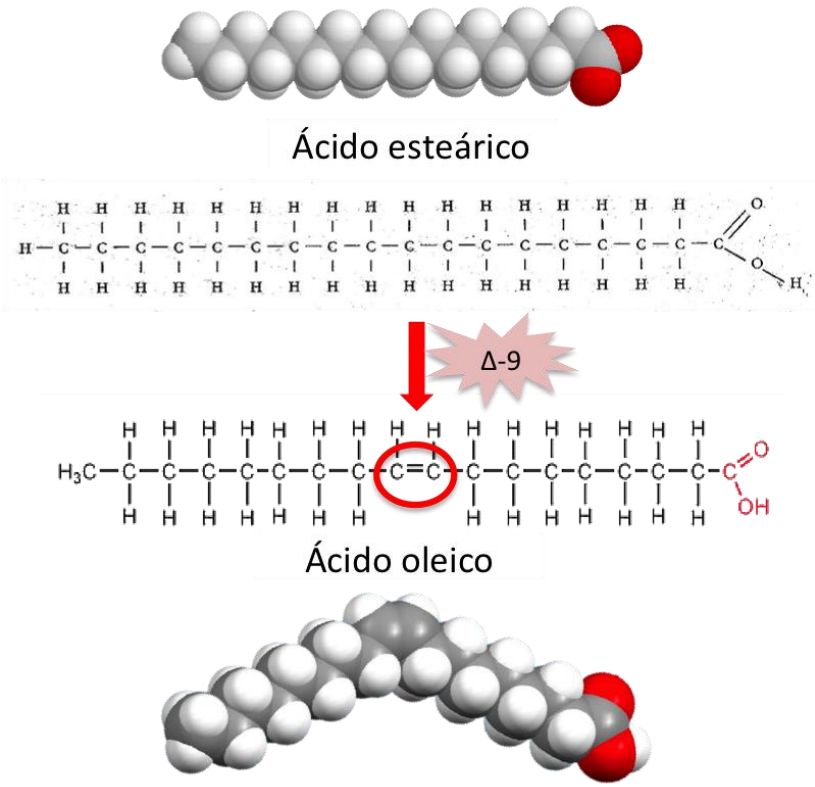
Numerosos factores, como el estado fisiológico y hormonal así como los ingredientes incluidos en la ración influyen en la actividad y expresión génica de las enzimas desaturasas (Zolfaghari y Ross, 2003). Entre ellos, la VA y su metabolito activo, el AR, parecen jugar un papel importante en la regulación de las desaturasas, más concretamente la estearoil-CoA-desaturasa o delta-9-desaturasa (Δ-9) y la delta-5-desaturasa.

La delta-5-desaturasa cataliza la conversión del 20:3 n-6 en 20:4 n-6 (ácido araquidónico) y del C20:4 n-3 en C20:5 n-3 (eicosapentanoico o EPA). Zolfaghari y colaboradores (2001) detectaron que la concentración de mRNA de la delta-5-desaturasa era tres veces superior en el hígado de ratas deficientes en VA, por lo que un aumento del nivel de VA en la ración puede reducir la actividad de esta enzima y como consecuencia las concentraciones de ácidos araquidónico y EPA, aunque por el momento esta afirmación no ha sido demostrada en las especies animales de interés zootécnico.

La delta-9-desaturasa o estearoil-CoA-desaturasa (SCD) es la enzima encargada de catalizar la reacción de desaturación inicial de los ácidos grasos de cadena larga a AGMI. El ácido palmítico (C16:0) y el esteárico (C18:0) (Figura 21) son los principales sustratos sobre los que actúa esta enzima convirtiéndolos en ácido palmitoleico (C16:1 n-7) y oleico (C18:1 n-9) respectivamente. Estudios previos han relacionado el nivel de suplementación de VA y la expresión y/o actividad de SCD, con resultados contradictorios. Numerosos estudios han observado un aumento en la actividad de la enzima (estimado mediante el índice de desaturación, que se determina por la relación C16:1/C16:0 y C18:1/C18:0) asociado a la restricción de VA en ganado vacuno (Siebert et al., 2006; Gorocica-Buenfil et al., 2008) y porcino (Olivares et al., 2009a; Olivares et al., 2011). Sin embargo, otros estudios han concluido que la VA no ejerce ningún efecto sobre el perfil de desaturación de la grasa (Gorocica-Buenfil et al., 2007c). También se ha observado una relación

positiva entre los niveles de VA y el grado de desaturación de la grasa en corderos (Daniel et al., 2004) y de la expresión de la enzima SCD en ratones (Miller et al., 1997).

Figura 21: Efecto de la enzima delta-9-desaturasa (Δ-9) sobre la formación de un doble enlace en la cadena carbonada del ácido esteárico



Se desconoce el mecanismo exacto por el que el AR afecta a la expresión de esta enzima, aunque se han planteado distintas hipótesis. En primer lugar, se describió que un agonista del gen *PPARG*, las tiazolidinedionas eran capaces de aumentar la expresión de la enzima desaturasa, estableciéndose por tanto, una relación entre dicha enzima y el gen *PPARG* (Kurebayashi et al., 1997). Sin embargo, más recientemente, se ha propuesto al *PPARA* como gen inductor de la expresión de *SCD* (Nakamura y Nara, 2002) y a los receptores nucleares *RAR* y *RXR* como mediadores de la acción de AR sobre el gen *SCD* (Samuel et al., 2001). Estos tres modos de acción tienen en común una estrecha relación entre el AR y cualquiera de los genes reguladores de la transcripción de *SCD* descritos.

1.5.2.7- Niveles de inclusión de vitamina A en el pienso y su efecto sobre la grasa intramuscular.

Una vez descritos los procesos en los que participa la VA, parece evidente la necesidad de asegurar un aporte suficiente de VA en la ración con el fin de cubrir las necesidades y así evitar carencias que puedan dar lugar a alteraciones del estado sanitario o a una disminución en la capacidad productiva de los animales. Sin embargo, niveles excesivos pueden acarrear problemas de toxicidad, resultan antieconómicos y pueden contribuir a la contaminación ambiental. Por ello, es imprescindible conocer las necesidades de VA (y de cualquier otro nutriente) del ganado porcino a lo largo de las distintas fases de producción. Con este objeto, el National Research Council (NRC) publica periódicamente las necesidades de nutrientes para las distintas especies de abasto, incluido el cerdo, basándose en la literatura científica. De este modo, se establecen unos niveles o necesidades mínimas, suficientes para evitar estados de deficiencia (Tabla 5) que sin embargo, tienden a ser ampliamente superados en la práctica productiva. Son varias las razones que justifican el mayor aporte de VA. Por un lado, existe un beneficio adicional sobre la salud que se refleja potencialmente en la productividad de los animales. Por otra parte, la suplementación en exceso sirve para compensar las variaciones que pueden darse en la práctica (distinta disponibilidad según el alimento, pérdidas en tratamientos por calor, en almacenamiento, variaciones en el consumo de pienso, cambios de manejo, calidad ambiental, estado sanitario, etc) (Hernández, 2002). La Tabla 5 presenta los niveles de VA en el pienso recomendados por el NRC (1998; 2012), así como por FEDNA y, en último lugar, los resultados obtenidos en una revisión sobre los contenidos de vitaminas en los piensos utilizados en España (Fraga y Villamide, 2000), en la que se analizó la composición vitamínica de 89 correctores utilizados, aproximadamente, en el 65% de las explotaciones porcinas españolas. Este estudio aporta una visión real de los niveles de suplementación habitualmente manejados en la industria de la alimentación animal, con el fin de mejorar los parámetros productivos.

Tabla 5: Niveles de suplementación de vitamina A (UI/kg pienso) recomendados por el National Research Council (NRC), por la Fundación Española para el Desarrollo de la Nutrición Animal (FEDNA) y niveles medios incorporados en piensos comerciales. (* Fuente: Fraga y Villamide, 2000)

	Lechones	Crecimiento/cebo	Gestación
NRC 1998	2200	1300	4000
NRC 2012	1879	1317	3847
FEDNA	13000	7500	6000
Niveles medios*	13800	7800	11765

Sin embargo, bajo ciertas circunstancias, una suplementación elevada puede producir efectos negativos sobre la productividad. Como se ha comentado en el apartado anterior, la VA tiene un efecto modulador sobre tres aspectos del metabolismo lipídico: la adipogénesis, la lipogénesis y la desaturación de ácidos grasos. El creciente interés de los consumidores y, por lo tanto, de la industria por la calidad de la carne ha conducido al desarrollo de diversos estudios que han investigado el efecto de la inclusión de distintos niveles de VA en el pienso sobre la cantidad de GIM (Tabla 6) y la composición de la grasa (Tabla 7).

Tabla 6: Estudios previos sobre el efecto de la inclusión de vitamina A (VA; UI/Kg alimento) en la dieta sobre el contenido en grasa intramuscular (GIM) en animales de abasto.

Especie	Rango pesos (kg)	Duración (Días)	Concentración VA	GIM (%)	Referencia
Vacuno, Holstein	218.4 -584.5	243	2,200 0	**4.2**$_a$ **5.6**$_b$	Gorocica-Buenfil et al., 2007c
Vacuno, Angus	224 -535.2	216	2,200 0	4.7 5	Gorocica-Buenfil et al., 2008
Vacuno, Angus	354.9 -679.8	268	600^c 0	**9.6**$_a$ **13**$_b$	Siebert et al., 2006
Ovino	28.7 -61	112	6,600 0	**3.88**$_b$ **3.09**$_a$	Arnett et al., 2007
Porcino, LWxLDxDU	23.7 -105	91	90 0	**1.30**$_a$ **2-00**$_b$	D'Souza et al., 2003
Vacuno, Holstein	252 -453	140	4,670 1,310	3,21 3,96	Marti et al., 2011
Porcino, DUx (LWxLD)	67.9 -125.9	57	7,500 100,000	3.42 3.75	Olivares et al., 2009a
Porcino, DUx (LWxLD)	56.4 - 114.5	55	0 100,000	**3,20**$_a$ **4.00**$_b$	Olivares et al., 2009b
Porcino, LWxLD	56.4 - 114.5	55	0 100,000	3.00 2,70	Olivares et al., 2009b
Porcino, LW x LD	55.8 -125.7	77	13,000 1,300 13,000-0	**1.98**$_a$ **2.52**$_b$ **2.09**$_{ab}$	Olivares et al., 2011

LW: Large White
LD: Landrace
DU: Duroc
PV: Peso vivo
a, b: letras diferentes representan diferencias significativas de contenido en GIM.
c: UI/kgPV

Como observamos en la Tabla 6, los resultados obtenidos son heterogéneos. Entre los factores que pueden alterar el efecto de la VA sobre el contenido en GIM, podemos destacar:

- La edad es un factor importante puesto que el efecto fundamental de la VA es consecuencia de su acción reguladora sobre la diferenciación adipocitaria o adipogénesis. Por tanto, las estrategias relacionadas con el contenido en VA de la dieta podrían ser especialmente eficaces a edades tempranas, cuando la adipogénesis es más activa y la proporción de preadipocitos en los depósitos grasos es mayor. Sin embargo, apenas existen estudios que comparen los efectos de la edad de suplementación de VA sobre el metabolismo lipídico.
- El sexo también parece ser un factor a tener en cuenta; en un estudio realizado con machos enteros y castrados (Marti et al., 2011), se ha observado un efecto superior de la restricción de VA sobre el contenido en GIM en los animales castrados (33.6%) que en los enteros (9%); este hecho podría ser consecuencia del diferente potencial adipogénico en ambos grupos, ya que los machos castrados también presentaron mayor cantidad de GIM (4.3% vs 2.8%).
- Se ha observado que la inclusión de VA puede tener efectos variables dependiendo de la especie en estudio. En corderos, la suplementación con VA produjo un aumento significativo de la GIM (Arnett et al., 2007); sin embargo el efecto contrario ha sido observado en vacuno, tanto de carne como lechero (Siebert et al., 2006; Gorocica-Buenfil et al., 2007c), aunque otros estudios no corroboran estos resultados en vacuno de carne (Gorocica-Buenfil et al., 2008) ni en machos Holstein, en los que sólo se observó una tendencia positiva de la restricción de VA sobre la GIM (Marti et al., 2011).

En la especie porcina, la bibliografía es escasa y los resultados controvertidos. Se ha observado que la restricción de VA provoca un aumento en la cantidad de GIM (D'Souza et al., 2003; Olivares et al., 2011), pero también que el nivel de inclusión de VA no ejerce ningún efecto sobre dicho parámetro (Olivares et al., 2009a).

Las diferencias observadas pueden deberse a la variabilidad en los tratamientos aplicados, el tiempo de retirada o la edad del animal. En el estudio en el que no se observaron diferencias en la cantidad de GIM, el tratamiento con menor nivel de inclusión de VA contenía 7,500 UI, lo cual es suficiente para cubrir las necesidades del animal según el NRC (2012) y por lo tanto no podría considerarse una restricción. La suplementación, por otro lado, no ha demostrado tener efectos contrarios a la restricción en este estudio (Olivares et al., 2009a), si bien en otro ensayo realizado por el mismo grupo y en cerdos con la misma genética (Du x (LD x LW), se observó que la GIM aumenta (4% vs 3.2%) cuando los animales son suplementados a niveles muy altos (100,000 UI/kg MS) respecto al grupo alimentado con 0 UI/kg MS (Olivares et al., 2009b). Además se ha observado en el citado estudio, una interacción entre genotipo y respuesta a la inclusión de VA, presentando los animales con genética paterna Duroc un cambio en el contenido en GIM (3.2% vs 4%) que no fue observado en los animales más magros (LD x LW) (Olivares et al., 2009b). Puesto que el efecto

de la VA está mediado principalmente por la regulación de la adipogénesis, la diferencia entre ambos genotipos puede deberse a diferencias en la precocidad entre ellos (Bonet et al., 2003; Gorocica-Buenfil et al., 2007c). Por tanto, el momento de aplicación del tratamiento es un factor que de nuevo merece ser estudiado en profundidad.

Tabla 7: Estudios previos sobre el efecto de la inclusión de vitamina A (VA) en la dieta sobre el perfil de ácidos grasos en distintos tejidos de animales de abasto.

Especie	Tejido	Duración	Nivel VA	AGS	AGMI	AGPI	Referencia
Vacuno, Angus	Grasa sc	268	600	**47$_b$**	**51,6$_a$**	-	Siebert et al., 2006
			0	**43,9$_a$**	**54,4$_b$**	-	
Vacuno, Holstein	Grasa sc	243	2.200	48	46	2.4	Gorocica-Buenfil et al., 2007c
			0	46	48	2.5	
Vacuno, Angus	Grasa sc	216	2.200	**48,7$_b$**	**39,9$_a$**	6.53	Gorocica-Buenfil et al., 2008
			0	**47,1$_a$**	**41,7$_b$**	6.68	
Porcino, DUx (LW x LD)	Grasa sc interna	57	100000	**43.7$_b$**	**43.8$_a$**	12.5	Olivares et al., 2009a
			7500	**42.2$_a$**	**44.9$_b$**	12.9	
Porcino, DUx (LW x LD)	GIM, LN	57	100000	39.7	54.5	5.7	Olivares et al., 2009a
			7500	38.9	55.6	5.5	
Porcino, LW x LD	Grasa sc externa	77	13000	**34.1$_a$**	40	25.9	Olivares et al., 2011
			1300	**35.5$_b$**	38.8	25.7	
			13000-0	**33.8$_a$**	39.2	27	
Porcino, LW x LD	GIM, LN	77	13000	37	45.1	17.9	Olivares et al., 2011
			1300	37.2	46.9	15.9	
			13000-0	37.4	46.2	16.4	
Porcino, LW x LD	Hígado	77	13000	**53.4$_b$**	21.2	**25.4$_b$**	Olivares et al., 2011
			1300	**50.4$_{ab}$**	17.7	**31.9$_a$**	
			13000-0	**49.1$_a$**	19.1	**31.81$_a$**	
Porcino, DUx (LW x LD)	GIM, LN	55	100000	**41.1$_a$**	**51.2$_b$**	7.5	Olivares et al., 2009b
			0	**39.0$_b$**	**53.2$_a$**	7.6	
Porcino, (LW x LD)	GIM, LN	55	100000	37.0	54.8	8.3	Olivares et al., 2009b
			0	38.1	54.1	7.6	

AGPI: Ácidos grasos poliinsaturados
Grasa sc: Grasa subcutánea
LW: Large White
LD: Landrace
DU: Duroc
PV:Peso vivo
a, b, c: letras diferentes representan diferencias significativas de contenido en GIM.

El resumen de algunos de los trabajos que han estudiado el efecto del nivel de inclusión de VA sobre el perfil de ácidos grasos muestra, de forma similar a lo ocurrido para el contenido en GIM, grandes discrepancias en los resultados (Tabla 7). Los factores que afectan a la respuesta del contenido en

GIM (como genética, dosis, edad o duración de la aplicación del tratamiento) podrían también explicar en buena medida la variabilidad en los resultados observados en cuanto a la composición de ácidos grasos. Además, parece ser que el tejido también condiciona enormemente la respuesta, como sugiere la variedad de resultados obtenidos en cerdo por Olivares y colaboradores (2011). En este estudio la respuesta a los distintos niveles de inclusión de VA varía desde un aumento de los AGS en la grasa subcutánea de animales alimentados con bajas dosis de VA, hasta una disminución de AGS en el hígado de estos mismos animales, mientras que la GIM no sufrió ninguna diferencia en su composición con distintos niveles de VA. Los mismos autores observaron en otro estudio realizado en cerdos más grasos (DUx (LWxLD)) sometidos a una restricción de VA, una disminución en la concentración de AGS y aumento de AGMI en los lípidos neutros de la GIM. Sin embargo, esta respuesta no se observó en cerdos magros (LWxLD) sometidos al mismo tratamiento (Olivares et al., 2009b). En rumiantes se ha visto también un efecto específico del tejido sobre el grado de desaturación de la grasa de animales alimentados con distintas concentraciones de VA. Daniel y colaboradores (2004) observaron en ovino que el aumento del nivel de VA en la ración generaba un aumento significativo de los contenidos de C16:1 n-7 y C18:1 n-9 en la grasa omental y una reducción de C18:0 y C16:0, aunque estos resultados no fueron observados en grasa subcutánea. De forma similar, Arnett y colaboradores (2007), también en corderos, observaron que los animales que habían recibido la dieta suplementada (6,600 UI/kg pienso) presentaban un contenido en ácido oleico en el músculo *Longissimus thoracis* significativamente mayor (41,2% vs. 38,3%) que los que no habían sido suplementados. En el hígado la respuesta de los animales al tratamiento fue la contraria (14,1% vs. 15%).

La gran variabilidad de resultados y de factores externos que afectan a la relación VA – perfil de ácidos grasos y VA – contenido en GIM ponen de manifiesto la dificultad en la extrapolación de estos resultados a nuevas razas o sistemas productivos, así como la necesidad de realizar nuevos estudios que aporten más datos al respecto. Debido a todo ello, parece especialmente interesante el uso de razas y sistemas productivos en las que el incremento de la GIM y el aumento de la actividad de la enzima SCD puedan ser relevantes, como es el caso de la raza ibérica, en la que los distintos sistemas productivos generan gran variabilidad en aspectos productivos y de calidad de carne.

2- PLANTEAMIENTO Y OBJETIVOS

El cerdo ibérico es una raza que se caracteriza por bajos rendimientos productivos y reproductivos, un crecimiento lento y muy diferente al de las razas seleccionadas, por su escaso desarrollo muscular, por su gran capacidad de acumulación de grasa y por su alta capacidad de adaptación al medio y rusticidad. Por ello, la cría de este cerdo, como ocurre generalmente con las razas autóctonas tradicionales, es poco eficiente, lo que suele ir asociado con escasos beneficios para el productor y por lo tanto con una disminución en el censo de cabezas que, en muchas ocasiones, llevan a la desaparición de la raza. Este no es el caso del cerdo ibérico, que cuenta con una diferencia esencial: su altísima calidad de carne, que se refleja en los altos precios que alcanzan sus productos y que compensan su menor rendimiento productivo. Por ello, de forma tradicional el sector se ha preocupado por mantener e incluso mejorar esta característica propia de la raza. Los factores que determinan la calidad de la carne derivan de las características de la raza y del sistema productivo en extensivo (López-Bote, 1998). Entre ellos, destaca por su interés la cantidad de GIM y su composición (principalmente el contenido en AGMI), así como la estabilidad oxidativa, la alta concentración tisular de vitamina E y la concentración de pigmentos hemáticos (Ventanas et al., 2006). La cantidad de GIM, así como su composición son parámetros muy variables, determinados en gran parte por la genética. Los fenotipos de menor calidad y mayor variabilidad para estos caracteres se observan principalmente en poblaciones cruzadas que, como se ha mencionado anteriormente, son las mayoritarias en cuanto a número de individuos. Además de la raza, otros factores como la nutrición, la epigenética o la edad también modifican la cantidad y composición de la GIM en cerdos. Por ello, se han realizado numerosos estudios en cerdo ibérico valorando el efecto que el sistema de producción, la alimentación, el sexo, el peso al sacrificio o el cruzamiento con otras genéticas podría tener sobre la cantidad y composición de GIM (Daza et al., 2007; Serrano et al., 2008; Rodriguez-Sanchez et al., 2010; Robina et al., 2013; Ayuso et al., 2014; Fuentes et al., 2014).

Debido a la necesidad de mantener la calidad de la carne tras la incorporación de genética Duroc a la raza ibérica, los efectos de este cruzamiento se han estudiado desde un punto de vista fenotípico (Ventanas et al., 2007; Serrano et al., 2008; Fuentes et al., 2014), así como desde un enfoque más profundo en cuanto a mecanismos moleculares y genéticos implicados (Pena et al., 2013; Óvilo et al., 2014). El empleo de técnicas de análisis del transcriptoma, como los microarrays de expresión y la secuenciación masiva del ARN generan una enorme cantidad de información relativa a la expresión génica. Esta información, estudiada mediante programas de interpretación funcional, permite identificar genes, rutas metabólicas y factores de transcripción responsables de las diferencias observadas a nivel fenotípico entre cerdos ibéricos puros y cruzados, lo que ayuda a entender los mecanismos moleculares que se encuentran detrás de estas diferencias. Además, la técnica de RNA Seq permite identificar variables estructurales que puedan también asociarse con estos cambios fenotípicos. El conocimiento generado en este tipo de estudios, que podríamos

englobar dentro de la investigación de carácter básico, puede ayudar a medio plazo a seleccionar animales (especialmente de raza Duroc) que puedan tener variantes estructurales o patrones de expresión de ciertos genes o factores de transcripción más próximos al ibérico para caracteres de interés. Esto permitiría, por lo tanto, minimizar la pérdida de calidad observada en productos procedentes de animales cruzados.

Además de la raza, otros factores como el contenido en micronutrientes en la dieta podría también afectar al contenido y composición de la GIM. El efecto de los niveles de inclusión de VA sobre estos parámetros en cerdos ibéricos no ha sido estudiado hasta ahora. La VA posee conocidas propiedades antiadipogénicas, y su efecto podría estudiarse desde un enfoque nutrigenómico, entendiéndose como tal la parte de la genética nutricional que investiga los efectos de los nutrientes sobre el fenotipo que están vehiculados por cambios en la expresión génica (Corella y Ordovas, 2009). Así, la VA modificaría la expresión de genes involucrados en la regulación de procesos relacionados con la diferenciación adipocitaria y con el metabolismo lipídico y de la propia VA, alterando así la cantidad y composición de la GIM.

Además, esta vitamina liposoluble interfiere en la absorción y metabolismo de otras vitaminas similares, como la vitamina E, muy importante desde el punto de vista de la calidad y la estabilidad de la carne, por lo que resulta interesante investigar el efecto que distintos niveles de inclusión pueden tener sobre el contenido en vitamina E en los tejidos.

Los objetivos principales de la presente Tesis Doctoral han sido, pues:

1- Profundizar en el conocimiento de los mecanismos moleculares implicados en la regulación de caracteres fenotípicos de interés en el cerdo ibérico, fundamentalmente relacionados con la diferenciación adipocitaria y con otros factores que modifican el desarrollo de la GIM.

Para la consecución de este objetivo se estudió el efecto del tipo genético y de la edad sobre el transcriptoma muscular de cerdos ibéricos puros y cruzados con Duroc (Experimento 1), con el objetivo de identificar genes, funciones biológicas, rutas metabólicas y reguladores involucrados en las diferencias fenotípicas observadas entre ambos genotipos. Para ello, se utilizaron machos ibéricos puros y cruzados con Duroc sacrificados en dos etapas de desarrollo distintas (nacimiento y 4 meses de edad) en los que se analizaron diversos caracteres fenotípicos de interés así como el transcriptoma de dos músculos de alta relevancia económica, el *Longissimus dorsi* (en ambas edades) y el *Biceps femoris* (al nacimiento).

2- Evaluar la eficacia de la restricción de vitamina A en la dieta como posible estrategia nutricional para aumentar la cantidad y mejorar la composición de la GIM en el cerdo ibérico.

Para ello, se estudió el efecto de la suplementación (10,000 UI/kg pienso) y la restricción (0 UI/kg pienso) de VA sobre caracteres de interés, relacionados con el rendimiento productivo, el desarrollo adipocitario, el contenido y composición de la GIM y los niveles de acumulación tisular de vitaminas A y E (Experimento 2). También se estudió la expresión de un panel de genes candidato relacionados con los caracteres fenotípicos mencionados. Para el desarrollo de este objetivo, se llevó a cabo un experimento con cerdos ibéricos puros que fueron sometidos bien a una suplementación o a una restricción de VA, iniciada en dos etapas críticas para el desarrollo de la GIM (2 y 4 meses de edad) y sacrificados secuencialmente (a los 4, 8 y 11 meses de edad, aproximadamente).

3- RESULTADOS

3.1 CAPITULO 1: El análisis comparativo del transcriptoma muscular entre genotipos porcinos identifica genes y mecanismos reguladores asociados al crecimiento, el engrasamiento y el metabolismo.

Comparative analysis of muscle transcriptome between pig genotypes identifies genes and regulatory mechanisms associated to growth, fatness and metabolism.

Miriam Ayuso, Almudena Fernández, Yolanda Núñez, Rita Benítez, Beatriz Isabel, Carmen Barragán, Ana I. Fernández, Ana I. Rey, Juan F. Medrano, Ángela Cánovas, Antonio González-Bulnes, Clemente J. López-Bote, Cristina Óvilo.

PLOS ONE. 2015. En evaluación

Comparative analysis of muscle transcriptome between pig genotypes identifies genes and regulatory mechanisms associated to growth, fatness and metabolism.

Muscle transcriptome in Iberian pigs

Miriam Ayuso[1], Almudena Fernández[2], Yolanda Núñez[2], Rita Benítez[2], Beatriz Isabel[1], Carmen Barragán[2], Ana I. Fernández[2], Ana I. Rey[1], Juan F. Medrano[4], Ángela Cánovas[4¥], Antonio González-Bulnes[3], Clemente J. López-Bote[1], Cristina Óvilo[2*].

[1]Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense, Madrid, Spain

[2]Departamento de Mejora Genética Animal, INIA, Madrid, Spain

[3]Comparative Physiology Lab SGIT-INIA, Madrid, Spain

[4]Department of Animal Science, University of California Davis, Davis, California, United States of America

[¥]Current affiliation: Department of Animal and Poultry Science. University of Guelph, Guelph, Center for Genetic Improvement of Livestock, Ontario

*Corresponding author: ovilo@inia.es (CO)

Keywords: transcriptome;RNA-Seq; differential expression; Iberian pig;muscle; regulatory mechanisms; growth; lipid metabolism; fat deposition; SNPs

3.1.1- Abstract

Iberian ham production includes both purebred (IB) and Duroc-crossbred (IBxDU) Iberian pigs, which show important differences in meat quality and production traits, such as muscle growth and fatness. This experiment was conducted to investigate gene expression differences, transcriptional regulation and genetic polymorphisms that could be associated with the observed phenotypic differences between IB and IBxDU pigs. Nine IB and 10 IBxDU pigs were slaughtered at birth. Morphometric measures and blood samples were obtained and samples from *Biceps femoris* muscle were employed for compositional and transcriptome analysis by RNA-Seq technology. Phenotypic differences were evident at this early age, including greater body size and weight in IBxDU and greater *Biceps femoris* intramuscular fat and plasma cholesterol content in IB newborns. We detected 150 differentially expressed genes between IB and IBxDU neonates ($p < 0.01$ and Fold-Change > 1. 5). Several were related to adipose and muscle tissues development (*DLK1, FGF21* or *UBC*). The functional interpretation of the transcriptomic differences revealed enrichment of functions and pathways related to lipid metabolism in IB and to cellular and muscle growth in IBxDU pigs. Protein catabolism, cholesterol biosynthesis and immune system were functions enriched in both genotypes. We identified transcription factors potentially affecting the observed gene expression differences. Some of them have known functions on adipogenesis (*CEBPA, EGRs*), lipid metabolism (*PPARGC1B*) and myogenesis (*FOXOs, MEF2D, MYOD1*), which suggest a key role in the meat quality differences existing between IB and IBxDU hams. We also identified several polymorphisms showing differential segregation between IB and IBxDU pigs. Among them, non-synonymous variants were detected in several transcription factors as *PPARGC1B* and *TRIM63* genes, which could be associated to altered gene function. Taken together, these results provide information about candidate genes, metabolic pathways and genetic polymorphisms potentially involved in phenotypic differences between IB and IBxDU pigs associated to meat quality and production traits.

3.1.2- Introduction

The pig is the main species for meat consumption worldwide, 43% of total produced meat comes from pigs. Most production comes from the modern European pig breeds, which have been extensively selected and show optimized productivity and efficiency (Chang et al., 2003). In the Mediterranean basin, there is also a significant production of unique high-quality traditional pork products from local breeds. The Mediterranean breeds, also known as fatty-pig breeds, have an ancient origin, and have been reared in extensive conditions for centuries, exposed therefore to harsh environments and seasonal variations in food availability (associated with the development of a thrifty genotype (Astiz et al., 2014)). These breeds are smaller in size, have not undergone

intense genetic selection and are less productive than modern breeds. As a consequence of the industrialization of pork production, three-quarters of the traditional breeds are extinct or marginalized (Murgiano et al., 2010). The exception is the Iberian pig, the most representative Mediterranean traditional breed, which has an important commercial value based on high quality dry-cured products in terms of consumers' health and acceptance (López-Bote, 1998).

Peculiarities in Iberian pig metabolism drive its valued meat properties; Iberian pigs are characterized by higher fat deposition, fat desaturation and food intake (Ovilo et al., 2005; Muñoz et al., 2009), as well as by higher circulating leptin levels in plasma (Fernandez-Figares et al., 2007) than lean pigs, suggesting a syndrome of leptin resistance. Moreover, the Iberian pig is also considered an amenable and robust biomedical model for obesity and associated cardiometabolic diseases since, when provided high levels of food, the animals are prone to the development of dyslipidemias, metabolic syndrome and type-2 diabetes (Torres-Rovira et al., 2012). On the other hand, as observed in other traditional breeds, productive performance is considerably lower than that of highly selected modern breeds. To improve reproductive and growth performances and primal cuts yield, in the last decades Duroc breed was introduced as terminal sire cross. Recently, Spanish law has accepted and regulated the use of Iberian X Duroc pigs to obtain "Iberian" products.

However, the introduction of Duroc genetics is associated with a decrease in meat quality, mainly determined by a decrease in intramuscular fat (IMF) and monounsaturated fatty acids (MUFA) contents (Ventanas et al., 2006). Intramuscular fat content and fatty acid composition are the main factors affecting meat quality and are highly dependent on genetic type and diet (Wood et al., 2008). Intramuscular fat content is determined both by number and size of adipocytes within muscle fibers. During prenatal development and immediately after birth, muscle fiber and preadipocyte differentiation are very active processes that slow down with animal growth (Sepe et al., 2011). Later in growth adipocyte hypertrophy is the most important issue affecting IMF content, although hyperplasia is maintained in the adult animal to a lesser extent (Gregoire et al., 1998). Thus, birth is a critical time-point to investigate muscle growth, adipocyte differentiation and metabolism, in which environmental effects are minimized. On the other hand, IMF composition and fatty acids profile depend on lipogenesis and fatty acids metabolism. It has been reported that breed affects adipogenesis, lipogenesis and their timing, as well as the expression patterns of adipocyte differentiation-related genes (Li et al., 2012). In this sense, Iberian pig is considered a more precocious breed than Duroc pig (Ovilo et al., 2014b).

Due to the influence of the genetic background on productive and meat quality traits, research in the past few decades has been focused on understanding the genetic basis of cell growth and development, myogenesis and metabolism (Ropka-Molik et al., 2014). Recently, new interest has arisen towards the understanding of genetic mechanisms underlying lipid synthesis and

accumulation, due to its importance in meat quality (Li et al., 2012). Different approaches such as candidate gene expression studies or cDNA microarray analysis have been used to investigate genetic aspects of target parameters. Some studies based on the microarray technology investigated transcriptome differences among Iberian pig and Large White or Duroc pig in endocrine tissues (Pérez-Enciso et al., 2009) and between Iberian and Iberian X Duroc crossbred pigs in *Longissimus dorsi* muscle (Ovilo et al., 2014b).

Currently, the availability of the RNA-Seq technology has allowed the assessment of global changes in transcriptome of a number of species including pigs (Ropka-Molik et al., 2014), because of its greater accuracy and reproducibility than microarray technology (Marioni et al., 2008; Wickramasinghe et al., 2014). RNA-Seq allows measuring not only gene expression, but also examining genome structure identifying SNP and other structural variation such as indel and splice variants. Some applications of this technology include transcript quantification, allele-specific expression, novel transcript discovery or single nucleotide polymorphism (SNP) discovery (Qian et al., 2014). In pigs, several RNA-Seq studies have been carried out for assessing differences in the transcriptome of muscle, fat, liver or hypothalamus among breeds or phenotypically extreme individuals within a breed for characters of interest (Pérez-Montarelo et al., 2014; Ropka-Molik et al., 2014; Ghosh et al., 2015). The RNA-Seq technology is still scarcely applied to the Iberian breed, with studies comprehending the assessment of phenotypically extreme individuals for fatty acids composition (Puig-Oliveras et al., 2014) or the exploration of gonad transcriptome in Iberian and Large White pigs (Esteve-Codina et al., 2011). However, to the best of our knowledge, there are not RNA-Seq technology-based studies focused on genetic differences between Iberian and other breeds aimed at improving meat quality and productive traits.

Meat quality in Iberian pigs is of special interest for carcass cuts used in the dry curing industry such as the loin and the ham. A previous study assessed transcriptomic differences between pure Iberian and Duroc-crossbred Iberian pigs using microarray technology in the loin (Ovilo et al., 2014b), but no information on ham muscles transcriptome exists. It is well known that different muscles differ in developmental timing, metabolic and physicochemical properties, including different responses to exercise (Te Pas et al., 2011). *Biceps femoris* (BF) muscle is the biggest muscle in the ham and shows higher oxidative capacity, and lower drip loss than *Longissimus dorsi* muscle (Karlsson et al., 1993; Lefaucheur et al., 2011). Moreover, important differences exist regarding the IMF content of both muscles. Karlsson et al. (1993) reported higher IMF content in LD muscle in Yorkshire pig breed, whilst the opposite was reported for Iberian pigs, where BF showed remarkably greater IMF content than several others carcass muscles (Ayuso et al., 2015a). Also, transcriptomic and proteomic comparisons between muscles showed important functional differences, with 15 – 30% of proteome differing between LD and BF (Te Pas et al., 2011; Herault et al., 2014). On the other hand, transcriptomic studies performed sequentially along early

development suggest the perinatal as a critical period to study genes affecting muscle and adipose cells growth and muscle fiber differentiation (Zhao et al., 2011; Gosh et al., 2015), in agreement with the tissue differentiation timing commented previously. Moreover, environmental effects are minimized at this time point.

Hence, in agreement with previous considerations, the present study was carried out to study the BF muscle of newborn piglets in IB and in the IBxDU cross, aiming to: 1) Verify whether phenotypic differences are evident from the very early developmental stages (newborns) in these closely related populations; 2) Evaluate changes in gene expression *in BF* muscle that may be responsible for the observed phenotypic differences and identify pathways and networks in which those genes are involved; 3) Identify transcription factors affecting gene expression in order to establish potential new candidate genes affecting productive parameters and meat quality; 4) Identify structural variants in these candidate genes, potentially involved in the observed expression differences.

These results are useful for the understanding of genetic pathways affecting pork production and may be also of translational value for the understanding of ethnic differences in obesity and associated disorders in lipid metabolism in human medicine.

3.1.3- Materials and methods

Ethics statement

Animal manipulations were done in compliance with the regulations of the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 about the protection of animals used in research. The experiment was specifically assessed and approved (report CEEA 2010/003) by the INIA Committee of Ethics in Animal Research, which is the named Institutional Animal Care and Use Committee (IACUC) for the INIA.

Animals and sample collection

Ten pure Iberian sows raised in the same commercial farm were employed at their third gestation cycle. All females were managed in the same conditions. Five sows were mated to Iberian boars and five to Duroc boars. At birth, nine pure Iberian (IB) and 10 Iberian x Duroc (IBxDU) male piglets were randomly selected from the ten litters (two from each litter excepting one litter providing just one Iberian male). Blood samples were collected from newborns in sterile heparin blood vacuum tubes (Vacutainer Systems Europe, Meylan, France). Immediately after recovery, the blood was centrifuged at 1500g for 15min and the plasma was separated and stored into polypropylene vials at −20°C until assayed for determination of glucose and lipids metabolism-indicating parameters. After blood collection, piglets were slaughtered. Several body development measures were

obtained with a measure-tape: total body length (from the rostral edge of the snout to the tail insertion), ham length (from the anterior edge of the *Symphysis pubica* to the *articulatio tarsi)*, total length of anterior and posterior limbs (from the distal edge of the hooves to the proximal edge of the *scapula* or *Symphysis pubica*, respectively) and thoracic, abdominal and ham circumferences. Carcasses were weighted and samples from BF muscle were vacuum-packed in low-oxygen permeable film and kept frozen at –20°C until fatty acid composition analysis. Prior to fatty acid analysis, muscle samples were freeze dried for two days in a lyophilizer (Lyoquest, Telstar, Tarrasa, Spain) and grounded in a Mixer Mill MM400 (Retsch technology, Haan, Germany) until muscle was completely powdered. For transcriptomic analysis, *BF* samples were immediately frozen in liquid nitrogen and maintained at –80°C until RNA extraction.

The metabolic status of the newborn piglets was evaluated. Glucose, fructosamine, triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) plasmatic levels were measured with a clinical chemistry analyzer (Saturno 300 plus, Crony Instruments s. r. l., Rome, Italy).

Tissue composition analysis

Biceps femoris muscle IMF content was quantified using the method proposed by (Segura and Lopez-Bote, 2014) based on gravimetrical determination of lipid content. Fatty acid methyl esters (FAMEs) were identified by gas chromatography as described by (Lopez-Bote et al., 1997) using a Hewlett Packard HP-6890 (Avondale, PA, USA) gas chromatograph equipped with a flame ionization detector and a capillary column (HP-Innowax, 30 m × 0.32 mm i.d. and 0.25 μm polyethylene glycol-film thickness). Results were expressed as grams per 100 grams of detected FAMEs.

Transcriptomic analysis

RNA extraction

A total of 12 animals were randomly selected to perform transcriptomic analysis, representing all available litters (6 animals of each genetic type). Total RNA was extracted from 50-100mg samples of *BF* muscle using the RiboPure TM of High Quality total RNA kit (Ambion, Austin, TX, USA) following the manufacturer's recommendations. RNA was quantified using a NanoDrop-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The quality of the RNA was evaluated using the RNA Integrity Number (RIN) value from the Agilent 2100 Bioanalyzer device (Agilent technologies, Santa Clara, CA, USA). The RIN values ranged from 7.5 to 9.8

Library construction and RNA sequencing

Sequencing libraries were made using the mRNA-Seq sample preparation kit (Illumina Inc., Cat. # RS-100-0801) according to manufacturer's protocol. Each library was sequenced using TruSeq SBS

Kit v3-HS, in paired end mode with the read length 2x76bp on a HiSeq2000 sequence analyzer (Illumina, Inc). Images from the instrument were processed using the manufacturer's software to generate FASTQ sequence files.

Mapping and assembly

Sequence reads were analyzed using CLC Bio Genomic workbench software 7.0 (CLC Bio, Aarhus, Denmark). Quality control analysis was performed using the NGS quality control tool, which assesses sequence quality indicators based on the FastQC-project (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Quality was measured taking into account sequence-read lengths and base-coverage, nucleotide contributions and base ambiguities, quality scores as emitted by the base caller and over-represented sequences (Cánovas et al., 2014). All the samples analyzed passed all the QC parameters having the same length (76 bp), 100% coverage in all bases, 25% of A, T, G and C nucleotide contributions, 50% GC on base content and less than 0.1% over-represented sequences. A hierarchical clustering of the samples was also performed. One IBxDU pig was discarded for further analysis because the sample deviated largely from the expected grouping in the clustering analysis, probably due to RNA sampling or processing problems. Sequence paired-end reads (76bp) were assembled against the annotated Sscrofa10.2 reference genome (http://www.ncbi.nlm.nih.gov/genome/?term=sus+scrofa) using the genome, annotated genes and mRNA tracks. Data was normalized by calculating the 'fragments per kilo base per million mapped reads' (FPKM) for each gene (Trapnell et al., 2010).

Differential expression analysis

The statistical analysis was performed using the total exon reads as expression values by the Empirical analysis of differential gene expression tool. This tool is based on the EdgeR Bioconductor package (Robinson et al., 2010) and uses count data (i.e. total exon reads) for the statistical analysis. Genes were filtered according to two criteria: a minimum mean group expression greater than 0.5 FPKM in at least one group and a Fold-Change (FC) of the expression differences between IB and IBxDU groups equal or higher to 1.5. Finally, those genes with a $p \leq 0.01$, corresponding to a false discovery rate (FDR) value ≤ 0.23, were considered as differentially expressed (DE).

Systems biology study

The biological interpretation of the DE genes observed in BF muscle was performed using three complementary approaches, in order to identify enriched GO terms, pathways and networks involving the DE genes, and potential regulators causing the observed changes in gene expression.

The enrichment analysis was carried out using the Wilcoxon test tool in the ConsensusPathDB database (Kamburov et al., 2011), available at the Max Plank Institute website, which provides batch enrichment analyses to highlight the most relevant GO terms associated to a gene list. Both, the list of genes overexpressed in IB and IBxDU were used. Functional terms with *p*-values lower

than 0.05 were considered enriched in the annotation categories. The *p*-values are corrected for multiple testing using the FDR method and are presented as *q*-values.

Additionally, Ingenuity Pathway Analysis, (IPA) (Ingenuity Systems, Qiagen, California) software was employed to identify and characterize biological functions, gene networks and canonical pathways affected by the DE genes.

Regulatory transcription factors (TRF), which could potentially affect the DE genes in the dataset were also studied by following complementary approaches. First, Regulatory Impact Factors (RIF1 and RIF2) metrics (Reverter et al., 2010; Hudson et al., 2012) were calculated for the whole set of DE genes obtained conditional on genetic type (150 genes). RIF1 assigns an extreme score to those TRF that are consistently most differentially co-expressed with the highly abundant and highly DE genes; RIF2 assigns an extreme score to those TRF with the most altered ability to act as predictors of the abundance of DE genes. Candidate TRFs in pigs were obtained from Animal TFDB (http://www.bioguo.org/AnimalTFDB/BrowseAllTF.php?spe=Susscrofa). A total of 1038 TRF were retrieved. Among them, 723 showed expression values greater than 0.5 FPKM in at least one experimental group and thus, were used in the RIFs metrics approach.

The RIF1 and RIF2 values were computed for the i^{th} TRF as follows:

$$RIF1_i = \frac{1}{n_{de}} \sum_{j=1}^{j=n_{de}} \hat{a}_j \, x \hat{d}_j \left(r1_{ij} - r2_{ij}\right)^2 \text{ and}$$

$$RIF2_i = \frac{1}{n_{de}} \sum_{j=1}^{j=n_{de}} \left[\left(e1_j x r1_{ij}\right)^2 - \left(e2_j x r2_{ij}\right)^2\right]$$

where n_{de} is the number of DE genes, a_j and d_j the estimated average expression and differential expression of the j^{th} DE gene, $r1_{ij}$ and $r2_{ij}$ the co-expression correlation between the i^{th} TRF and the j^{th} DE gene in each one of the genetic types and being $e1_j$ and $e2_j$ the expression of the j^{th} gene in each genetic type (Almudevar et al., 2006). Both RIF measures for each analyzed TRF were transformed to standardized *z*-scores by subtracting the mean and dividing by its standard deviation. We identified relevant TRF as those with extreme RIF z-scores according to the corresponding confidence intervals (CI) calculated by bootstrap. In each iteration of bootstrapping, a set of n_{de}= 150 genes were randomly selected from the 11392 expressed genes, and the RIF1 and RIF2 z-scores of the 723 TRF were calculated. The procedure was repeated 10,000 times for each scenario to obtain the corresponding 95 and 99% CI intervals of both z-scores.

Complementarily, IPA software was employed to identify and characterize potential regulators using two different tools, the *upstream regulators* and the *regulators* tools. Both of them identify known regulators that may be affecting expression of the dataset of DE genes. IPA-identified regulators include genes, but also other molecules as drugs. Thus, out of the identified regulators, only genes that were also included in the RIFs metrics candidate TRF list (which consisted of 723

TRF) were considered (genes included in the animal TFDB and with expression values higher than 0.5 FPKM in at least one experimental group).

Using the information obtained from the TRF study, an additional search for enriched pathways and networks was carried out with IPA software considering both, DE genes and TRF.

Structural variants analysis

A search of structural variants was performed by pooling the reads coming from all animals in each genetic type, and comparing the variants found in each group. The probabilistic variants detection tool (CLC Bio Genomic workbench) was used to perform the variant calling analysis. Single-end read alignments were not ignored. The minimum coverage in a locus to be considered was set up as 10 and the variant probability as 90. The variant probability parameter defines how good the evidence has to be at a particular site for the tool to report a variant at that location. Specifically, the variant probability threshold set as 90 means that any candidate variation in the genome must have a probability lower than 0.1 (1-0.9) of being the same as the reference sequence, to be considered as a variant. Variants with an allele frequency under 5% and/or coverage under 30 reads for the general variant analysis and under 10 reads in the candidate genes variant analysis were not considered. Variants were considered to be potentially fixed (frequency greater or equal to 90%) or segregating (frequency lower than 90 %).

The variants identified in genes corresponding to transcription factors (i.e. *EGR2*, *FOS*, *FOXO1*, *FOXO3*, *IRF1*, *STAT5B*, *HOXA9*, *ATF4*, *TP53*, *NOR-1*, *ABRA*, *ATF3* and *PPARGC1B*) were functionally evaluated using the variant effect predictor (VEP) tool from Ensembl (www.ensembl.org/info/docs/tools/vep/) which includes information about amino acid change localization and consequences, affected transcripts, and SIFT (Ng and Henikoff, 2003) and PolyPhen (Adzhubei et al., 2010) scores.

Results validation by RT qPCR

RNA obtained from the 11 animals under study was employed to perform the technical validation of the differential expression of some genes that were either upregulated in IB, upregulated in IBxDU or not DE between genetic types. This technical validation was performed by studying the Pearson correlation between the expression values obtained from RNAseq data (FPKM) and the normalized gene expression data obtained by RT qPCR. Moreover, RNA obtained from all the available animals (9 IB and 10 IBxDU) was used to quantify expression differences of such genes.

First-strand cDNA synthesis was carried out with Superscript II (Invitrogen, Life Technologies, Paisley, UK) and random hexamers in a total volume of 20 µl containing 1 µg of total RNA and following the supplier's instructions.

The expression of 9 genes was quantified by qPCR. Primer pairs used for quantification were designed using Primer Select software (DNASTAR, Wisconsin, USA) from the available GENBANK and/or ENSEMBL sequences, covering different exons in order to assure the amplification of the

cDNA. Sequence of primers and amplicon lengths are indicated in S1 Table. Standard PCRs on cDNA were carried out to verify amplicon sizes. Quantification was performed using SYBR Green mix (Roche, Basel, Switzerland) in a LightCycler480 (Roche, Basel, Switzerland), following standard procedures Data were analyzed with LightCycler480 SW1.5 software (Roche, Basel, Switzerland). All samples were run in triplicate and dissociation curves were carried out for each individual replicate. Single peaks in the dissociation curves confirmed the specific amplification of the genes. For each gene, PCR efficiency was estimated by standard curve calculation using four points of cDNA serial dilutions. Mean Cpvalues were employed for the statistical analyses of differential expression. Stability of four endogenous genes (i. e. *GAPDH*, *B2M*, *TBP* and *ACTB)* was calculated using Genorm software (Vandesompele et al., 2002) The *TBP* and *ACTB* genes were selected as the most stable endogenous genes to normalize the data. The qPCR expression data normalization was performed using normalization factors calculated with Genorm software. Relative quantities were divided by the normalization factors, which were the geometric means of the two reference genes quantities.

Statistical analyses of tissue composition and qPCR expression quantification

Phenotypic data were analyzed as a completely randomized design using the general linear model (GLM) procedure using SAS version 9.2 (SAS Inst. Inc., Cary, NC; 2009). The mean and genetic type were considered as systematic effects, and residual effects as random. Carcass weight was used as covariate when it was significant and removed from the model when it was insignificant. The animal was the experimental unit for all analysis. The results were considered to be significant at *p*-value < 0.05.

Statistical analysis of gene expression data was carried out following the method proposed by (Steibel et al., 2009) which consists of the analysis of cycles to threshold values (Cp), for the target and endogenous genes using a linear mixed model. The following model was used for analyzing the joint expression of the target and control genes in different tissues:

$$y_{gikr} = TG_{gi} + B_{gk} + D_{ik} + e_{gikr}$$

where, *Eg*is the efficiency of the PCR of *g*th gene, Cp_{gikr} is the value obtained from the thermocycler software for the *g*th gene from the *r*th replicate in a sample collected from the *k*th animal of the *i*th genetic type, TG_{gi} is the specific effect of the *i*th genetic type on the expression of gene *g*th, B_{gjk} is specific random effect of the *k*th pig on the expression of gene *g*th, D_{ijk} is a random sample-specific effect common to all the genes, and e_{gikr} is a residual effect.

To test differences in the expression rate of genes of interest ($diff_{TG}$) between classes normalized by the endogenous genes, different contrasts were performed between the respective estimates of *TG* levels. Significance of $diff_{TG}$ estimates was determined with the *t* statistic. To obtain FC values from the estimated $diff_{TG}$ values, the following equation was applied: .

P-values < 0.05 were considered statistically significant.

To validate the global RNA-Seq results, the concordance correlation coefficient (CCC) (Miron et al., 2006) was calculated between the FC values estimated in *BF* muscle from RNA-Seq and qPCR expression measures for the 9 genes analyzed by the two technologies (RNA-Seq and qPCR).

3.1.4- Results and discussion

Phenotypic differences between genetic types

The results obtained in the present study constitute the first assessment of phenotypic differences between IB and IBxDU piglets at birth. There are several studies evaluating phenotypic differences between both genotypes at weaning or adulthood (Ventanas et al., 2006; Serrano et al., 2008; Robina et al., 2013; Fuentes et al., 2014; Ovilo et al., 2014b). Pure Iberian and crossbred piglets were slaughtered at birth at an average of 1.2 and 1.8 kg live weight, respectively (SEM = 0.06). Genetic type affected all the carcass phenotypic parameters: IBxDU neonates were bigger and heavier ($p < 0.001$) than IB newborns (Table 1), reflecting previously reported differences in the same traits in adult animals (Serrano et al., 2008; Robina et al., 2013).

The assessment of differences in glucose and lipids metabolism (Table 1) showed that purebred IB piglets have greater plasma levels of total and HDL cholesterol, and triglyceride than IBxDU neonates. These differences at birth are concordant with the similar differences previously found between purebred Iberian and lean crossbred (Large White x Landrace x Pietrain) fetuses (Torres-Rovira et al., 2013). Cholesterol is of vital importance for the offspring as a key constituent of cell membranes and the precursor of hormones and metabolic regulators (Woollett, 2001; Palinski, 2009). Placental and fetal tissues have the capacity for *de novo* cholesterol synthesis (Wadsack et al., 2007) but the high demand from the fetuses makes the transport of maternal cholesterol through the placenta necessary (Herrera, 2002; Herrera et al., 2006). Triglycerides are also indispensable as a major source of energy for the developing fetus and are also transferred from maternal circulation (Szabo et al., 1973; Coleman and Haynes, 1984). Previous studies have found that adequate availability of cholesterol and triglycerides is even more critical in fatty-pigs breeds (Gonzalez-Bulnes et al., 2012b), which have higher values of plasma lipids indexes than lean breeds (Torres-Rovira et al., 2013).

These results reinforce that genetic differences between fatty-pigs and lean breeds are established from prenatal stages and, together with previous results, may also give evidence of a genetic predisposition for lipid metabolism alterations in the Iberian breed. The same findings regarding plasma cholesterol and triglycerides levels have been reported in humans with familial combined hyperlipidemia, the most common genetic form of hyperlipidemia in human (Mata et al., 2014; Luo et al., 2015) and in the Rapacz familial hypercholesterolaemic swine model.

Regarding the IMF content and composition in BF (Table 1), IB showed almost 30% higher IMF content than IBxDU piglets (p = 0.014). The genetic type affected IMF composition as well, IB pigs showing greater ∑n-6/∑n-3 ratio (p = 0.031) (mainly due to greater proportion of C18:2 n-6), and lower ∑SFA (saturated fatty acids) content (p = 0.035). Also, a trend for a higher oleic acid content was observed in IB pigs (p = 0.092). As reported in previous studies, crossing with Duroc sires decreased IMF concentration, in agreement with differences observed in adult pigs (Ventanas et al., 2006; Ovilo et al., 2014b).

The differences observed between IB and IBxDU in parameters such as body size and weight, lipid metabolism-related indicators or IMF were surprising taking in account the early stage of development. This highlights the importance of improving the knowledge on molecular aspects responsible for such phenotypic differences at very early ages with the dual purpose of improving production in local breeds with distinctive products and providing adequate models for human diseases.

Table 1: Carcass, *Biceps Femoris* and metabolism phenotypic characteristics in IB and IBxDU piglets

	Genetic type		SEM[3]	*p*-value
	IBxDU [1]	IB[2]		
Carcass characteristics				
Carcass weight, kg	1.41	0.96	0.05	0.0005
Ham weight, kg	0.16	0.11	0.00	0.0008
Total body lenght, cm	40.20	35.50	0.31	0.0004
Ham lenght, cm	7.45	6.33	0.12	0.0009
Forelimb lenght, cm	12.35	10.83	0.12	0.0042
Hindlimb lenght, cm	15.95	13.83	0.12	0.0016
Torax circumference, cm	25.15	22.06	0.14	0.0010
Abdomen circumference, cm	18.90	17.28	0.19	0.0486
Ham circumference, cm	12.55	10.89	0.15	0.0020
Lipid and glucose metabolism-related plasma indicators				
Cholesterol, mg/dl	62.19	102.36	5.60	0.0030
Fructosamine, mg/dl	169.70	133.67	10.37	0.1009
Glucose, mg/dl	132.40	123.44	10.80	0.6839
LDL[4], mg/dl	42.16	45.82	4.40	0.4496
HDL[5], mg/dl	22.38	41.20	4.25	0.0176
Triglycerides, mg/dl	30.00	76.67	5.11	0.0003
Biceps femoris muscle fatty acids composition (g/100 g total fatty acids)				
IMF[6], %	1.72	2.21	0.09	0.0142
C12:0	0.65	0.58	0.03	0.2321
C14:0	2.57	2.32	0.12	0.3189
C15:1	1.28	1.18	0.06	0.3762
C16:0	25.90	25.44	0.19	0.2379
C16:1 n-9	1.90	2.09	0.05	0.3854
C16:1 n-7	5.38	4.57	0.21	0.0773
C17:0	1.69	1.44	0.07	0.0814
C17:1	0.91	0.81	0.06	0.3858
C18:0	10.85	9.96	0.25	0.1014
C18:1 n-9	23.80	25.82	0.56	0.0921
C18:1 n-7	6.15	5.69	0.18	0.2163
C18:2 n-6	7.31	9.17	0.60	0.1395
C20:1 n-9	0.53	0.53	0.01	0.9701
C20:2 n-6	0.41	0.40	0.03	0.8331
C20:3 n-6	0.62	0.55	0.02	0.0293
C20:4 n-6	6.31	5.99	0.20	0.4264
C22:1 n-9	1.21	1.08	0.05	0.1956
C22:4 n-6	1.58	1.27	0.09	0.0925
C22:5 n-3	0.48	0.50	0.02	0.5405
C22:6 n-3	0.67	0.62	0.02	0.1959
∑SFA[7]	41.66	39.74	0.42	0.0350
∑MUFA[8]	41.01	41.77	0.40	0.3530
	Genetic type		SEM[3]	*p*-value
	Genetic type		SEM[3]	*p*-value
	IBxDU [1]	IB[2]		
∑PUFA[9]	17.34	18.49	0.50	0.2639
UI[10]	96.20	97.79	0.90	0.3904
∑n-3[11]	1.77	1.67	0.04	0.1940
∑n-6[12]	15.56	16.82	0.48	0.2089
∑n-6/∑n-3	8.78	10.17	0.30	0.0319
∑MUFA/∑SFA	0.99	1.05	0.02	0.0659

[1] IBxDU = Iberian x Duroc crossbred pigs (n=10)

[2] IB = Purebred Iberian pigs (n=9)
[3]SEM = Standard error of the mean
[4]LDL = Low density lipoproteins
[5]HDL = High density lipoproteins
[6]IMF = Intramuscular fat
[7]ΣSFA = Sum of saturated fatty acids
[8]ΣMUFA = Sum of monounsaturated fatty acids
[9]ΣPUFA = Sum of polyunsaturated fatty acids
[10]UI = Unsaturation index = 1 × (% monoenoics) +2 × (% dienoics) +3 × (% trienoics) +4 × (% tetraenoics) +5 × (% pentaenoics) +6 × (% hexaenoics) (Hulbert et al., 2007)
[11]Σn3 = Sum of n-3 fatty acids
[12]Σn6 = Sum of n-6 fatty acids

Identification of differentially expressed genes by RNA-Seq analysis

An average of approximately 79 million sequence reads was obtained for each individual sample; these were assembled and mapped to the annotated Sscrofa10.2 genome assembly (22,861 genes). In all samples, 67-77% of the reads were categorized as mapped reads to the porcine reference sequence.

The FPKM values were used to establish the total number of genes expressed in muscle transcriptome (>0.5 FPKM). Approximately 50 % of total porcine annotated genes in the Sscrofa10.2 genome assembly were expressed in the studied samples (an average of 11,392 genes out of 22,861 annotated genes).

Ninety-five genes were overexpressed in IB (FC ranging from 1.9 to 12) and 55 genes were overexpressed in IBxDU (FC ranging from 2 to 63. 5) ($p < 0.01$) (S2 Table).

Large expression differences were observed for an unidentified protein in pig (ENSSSCG00000026923; FC = 63.4x), and for the genes *MARCO* (27.2x) and *CXCL13* (27.1x), which showed greater expression level in IBxDU than in IB piglets. *MARCO* and *CXCL13* genes are both related to immune response. *MARCO* is also involved in cytoskeleton and cell morphology determination of certain immune cells (Granucci et al., 2003) and *CXCL13* has also been found to be upregulated in adipocytes when compared to preadipocytes (Kabir et al., 2014).

On the other hand, another unidentified protein (ENSSSCG00000023287; FC =12.5x), and the pig genes *CIART* (8.4x) and *ATF3* (7.8x) were upregulated in IB piglets at birth. *CIART* is a transcription repressor of the mammalian circadian clock that inhibits the activators *CLOCK* and *BMAL-1* (Annayev et al., 2014). The mammalian circadian clock regulates sleep–wake rhythms, body temperature, blood pressure, hormone production, immune system or cell cycle (reviewed in (Merbitz-Zahradnik and Wolf, 2015)). It is also important for energy homeostasis regulation, as multiple genes involved in nutrient metabolism and metabolically related hormones such as insulin or leptin display rhythmic oscillations (Fonken and Nelson, 2014). It has been reported that animals deficient in *BMAL-1* show altered lipid homeostasis (i.e. an increase in the levels of

circulating fatty acids, including triglycerides, free fatty acids, and cholesterol) and metabolic syndrome (Shimba et al., 2011), which is in agreement with the phenotypic results observed in IB pigs. *ATF3* codes for a transcription factor considered as an adaptation-response gene involved in a variety of processes such as immunity, regulation of the cell cycle and apoptosis (Thompson et al., 2009), and cellular stress response (Hai and Hartman, 2001).

Some other interesting DE genes are related to muscle and adipose tissue development, for example *SLC2A4*, *FGF21*, *UBC*, or *ACHE* (Wallberg-Henriksson and Zierath, 2001; Stevenson et al., 2003; Lin et al., 2010; Francesc et al., 2014). Moreover, three DE genes (*DLK1, MYH10* and *ZWILCH*) were also observed to be DE between IB and IBxDU in a previous study (Ovilo et al., 2014b), where *Longissimus dorsi* transcriptome was compared at weaning. *DLK1* is a transmembrane protein expressed in preadipocytes but not in mature adipocytes (Wang et al., 2010), thus, greater expression in IB neonates (2.6x) suggests greater number of undifferentiated preadipocytes in IB than in IBxDU piglets, possibly associated with a greater adipogenic potential. However, in a previous study, *DLK1* gene was found to be upregulated in IBxDU pigs at weaning (Ovilo et al., 2014b). The different age of sampling could explain the opposite results: IB piglets may be born with higher amounts of preadipocytes that differentiate faster than those from IBxDU pigs thus, leading to lower preadipocyte content at weaning age. Similarly, a different pattern for myocyte differentiation has been reported between high and low muscle growth efficiency breeds, such as Landrace, Lantang, Pietrain or Duroc (Cagnazzo et al., 2006; Zhao et al., 2011). In Landrace, myocyte differentiation develops faster than in low efficiency breeds (Cagnazzo et al., 2006; Zhao et al., 2011). In addition, the proliferation and differentiation of preadipocytes are stronger and faster in Bamei than in Landrace (representing a fat- and lean-type pig breed, respectively) (Wickramasinghe et al., 2014; Zhang et al., 2014). Thus, we suggest a faster adipocyte differentiation in IB pigs at an early age that may conclude earlier than in IBxDU pigs.

MYH10 gene codes for a heavy-chain myosin, and was also found upregulated in IBxDU pigs when compared to IB pigs at birth and at weaning (Ovilo et al., 2014b), which suggests a greater development of muscular cells in crossbreds. The gene *ZWILCH* is overexpressed in IB at both ages; it is involved in cell proliferation and differentiation, and it may also play a role in the control of adipogenesis (Hamam et al., 2014), thus being an interesting candidate to explain phenotypic differences in adipogenesis and lipogenesis.

In order to validate the results obtained from the RNA-Seq analysis, the relative expression of some DE genes (upregulated in both genetic types) and a few non-DE genes was assessed by qPCR in all the available samples. A concordance correlation coefficient was calculated (CCC = 0.93) and denoted a high general concordance between RNA-Seq and qPCR expression values (Miron et al., 2006). In general good individual correlation values were obtained (S3 Table). Fold-Change and

significance tended to be greater when expression differences were analyzed by RNA-Seq technology, in accordance with its higher sensitivity (Wang et al., 2009).

Biological interpretation of the differential expression results

Different approaches were used to perform an exhaustive and robust biological interpretation of the results obtained in the transcriptome study. Results obtained from the Gene Ontology (GO) term overrepresentation analysis, performed to detect active biological processes differing in both IB and IBxDU, are shown in Table 2. In addition, IPA software was used to find biological functions overrepresented in both genetic types (S4 Table and Figs. 1 - 3) and to identify pathways (Table 3) and networks (Fig. 4) associated with the DE genes.

Table 2: Gene Ontology (GO) overrepresented terms regarding the biological process category.

	*GO term	*p*-value	*q*-value	Term name
COMMON	GO:0007165	9.54E-07	6.96E-05	Signal transduction
	GO:0031323	3.81E-06	1.39E-04	Regulation of cellular metabolic process
	GO:0009059	6.10E-05	6.37E-04	Macromolecule biosynthetic process
	GO:0044267	1.22E-04	8.10E-04	Cellular protein metabolic process
	GO:0010646	2.44E-04	1.11E-03	Regulation of cell communication
	GO:0030154	2.44E-04	1.11E-03	Cell differentiation
	GO:0048584	7.81E-03	1.84E-02	Positive regulation of response to stimulus
IB[1]	GO:0010467	1.22E-04	8.10E-04	Gene expression
	GO:0043412	1.22E-04	8.10E-04	Macromolecule modification
	GO:0036211	2.44E-04	1.11E-03	Protein modification process
	GO:0009889	4.88E-04	1.98E-03	Regulation of biosyntheticprocess
	GO:0090304	9.77E-04	3.56E-03	Nucleic acid metabolic process
	GO:0019438	1.95E-03	5.48E-03	Aromatic compound biosynthetic process
	GO:0016070	1.95E-03	5.48E-03	RNA metabolic process
	GO:0023056	7.81E-03	1.84E-02	Positive regulation of signaling
IBxDU [2]	GO:0002684	1.95E-03	1.86E-02	Positive regulation of immune system process
	GO:0050790	7.81E-03	3.49E-02	Regulation of catalytic activity
	GO:0016337	7.81E-03	3.49E-02	Single organismal cell-cell adhesion

[1] IB = Purebred Iberian pigs; [2] IBxDU = Iberian X Duroc crossbred pigs

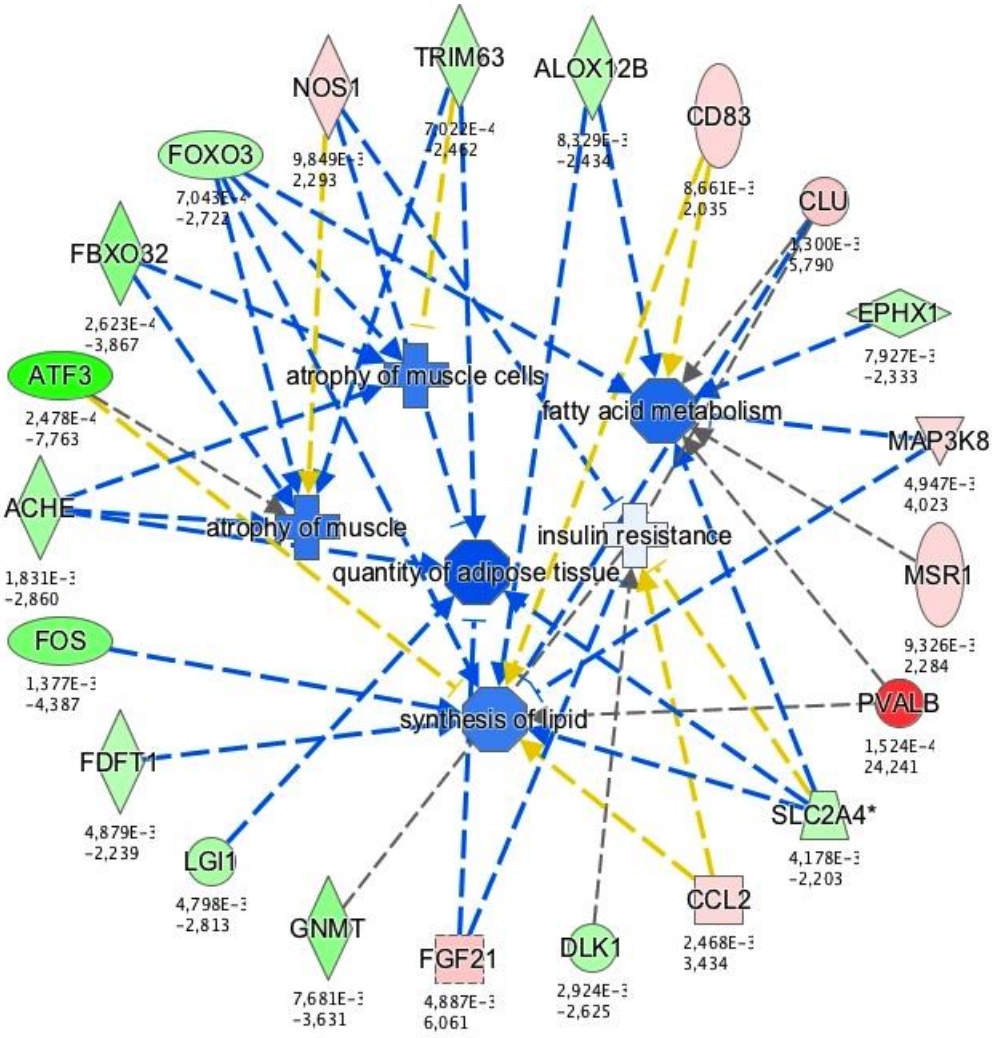
*GO terms are considered either common, when they are enriched in both genetic types and specific when the GO term is only enriched in one of the two genetic types.

Upregulated functions and pathways in *Biceps femoris* muscle from purebred Iberian piglets

Enriched biological functions in IB piglets identified by IPA software were mainly related with lipid and glucose metabolism (i.e. *Concentration of lipid*, *Synthesis of lipid*, *Insulin resistance*, *Quantity of adipose tissue* or *Fatty acid metabolism;* Fig. 1) and with muscle growth (S4 Table). In agreement, several DE genes with known functions related to lipid metabolism such as *FOS*, *FDFT1*, *SLC4A2*, *TRIM63, EPXH1, ALOX12B, FOXO3A* or *ACHE* were overexpressed in IB, possibly associated to the higher amount of adipose tissue observed in BF muscle of IB when compared to IBxDU pigs (Table 1). Similar results have been found in IB and IBxDU piglets at 28 days of age in loin muscle (Ovilo et al., 2014b). However, in the previous study, a different set of DE genes related to lipid metabolism was found, probably due to the high complexity of processes and molecular mechanisms regulating lipid metabolism at that stage of development. In another study comparing the muscle transcriptome of two divergent breeds for muscle and fat deposition, several muscle metabolism-related genes were identified as potential regulators of IMF deposition (Zhao et al., 2011), such as *FOS* and *ABRA* genes, identified also in the present study.

Fig 1: Enriched biological functions in IB pigs.

The network generated by IPA software shows enriched biological functions (blue color) and genes predicted to be involved in enrichment of these functions. Genes upregulated in IB pigs are highlighted in green color and genes upregulated in IBxDU pigs are highlighted in red color. Lines ending in an arrow represent activation; lines ending in a bar represent inhibition. Orange lines indicate activation of the biological function. Yellow lines represent findings inconsistent with the state of the biological function and grey lines a non-predicted effect.



Accordingly, several pathways enriched in IB pigs ($p < 0.05$) (Table 3) were related to the control of energy homeostasis (*Wnt/Ca+*, *Glutamine Biosynthesis* or *Fatty Acid α-oxidation* pathways) and to protein synthesis and cell growth (*Growth Hormone* (*GH) Signaling* and *IGF-1 Signaling*); IGF-1 is essential during prenatal and GH during postnatal growth (Butler and Roith, 2001). The effect of GH and IGF-1 on adipose tissue development and metabolism is controversial, as both have been proposed to play either a positive or a negative role on adipocyte differentiation (Wabitsch et al., 1995; Gerfault et al., 1999). Thus, in IB newborns, the activation of these pathways might be

associated to both muscle and preadipocytes development and differentiation. Accordingly, the *adipogenesis pathway* showed a trend for enrichment (p = 0.055).

Table 3: Pathways significantly enriched in Purebred (IB) and Duroc-crossbred (IBxDU) Iberian pigs.

IB		IBxDU	
Pathway	*p*-value	Pathway	*p*-value
PI3K Signaling in B Lymphocytes	<0.001	Agranulocyte Adhesion and Diapedesis	<0.001
NRF2-mediated Oxidative Stress Response	0.0022	LXR/RXR Activation	<0.001
Glutamine Biosynthesis I	0.0031	Atherosclerosis Signaling	<0.001
IL-17A Signaling in Fibroblasts	0.0051	Aldosterone Signaling in Epithelial Cells	0.0012
April Mediated Signaling	0.0060	Granulocyte Adhesion and Diapedesis	0.0019
LXR/RXR Activation	0.0060	TREM1 Signaling	0.0050
Epoxysqualene Biosynthesis	0.0062	Protein Ubiquitination Pathway	0.0054
B Cell Activating Factor Signaling	0.0066	Citrulline-Nitric Oxide Cycle	0.0071
Thyronamine and Iodothyronamine Metabolism	0.0091	IL-12 Signaling and Production in Macrophages	0.0155
Thyroid Hormone Metabolism I (via Deiodination)	0.0091	Superpathway of Citrulline Metabolism	0.0195
Wnt/Ca+ pathway	0.0129	nNOS Signaling in Skeletal Muscle	0.0209
Tight Junction Signaling	0.0145	Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	0.0251
Acute Phase Response Signaling	0.0151	Production of NO and Reactive Oxygen Species in Macrophages	0.0263
ERK5 Signaling	0.0158	Clathrin-mediated Endocytosis Signaling	0.0275
Growth Hormone Signaling	0.0191	Actin Cytoskeleton Signaling	0.0372
Clathrin-mediated Endocytosis Signaling	0.0191	Role of p14/p19ARF in Tumor Suppression	0.0417
Melatonin Signaling	0.0195	IL-17A Signaling in Fibroblasts	0.0468
IB		IBxDU	
Pathway	*p*-value	Pathway	*p*-value
Toll-like Receptor Signaling	0.0214		
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	0.0245		
UVA-Induced MAPK Signaling	0.0295		
Antioxidant Action of Vitamin C	0.0355		
IGF-1 Signaling	0.0355		
T Cell Receptor Signaling	0.0355		
Role of IL-17A in Psoriasis	0.0389		
Cholesterol Biosynthesis I	0.0389		
Cholesterol Biosynthesis II (24. 25-dihydrolanosterol)	0.0389		
Cholesterol Biosynthesis III (viaDesmosterol)	0.0389		
Vitamin-C Transport	0.0417		
Glucocorticoid Receptor Signaling	0.0457		
Fatty Acid α-oxidation	0.0479		

[1] IB = Purebred Iberian pigs; [2] IBxDU = Iberian X Duroc crossbred pigs

Upregulated functions and pathways in *Biceps femoris* muscle from Duroc-crossbred Iberian piglets

Enriched functions in IBxDU newborns (S4 Table) were related to cell growth and differentiation, such as *Recruitment and transmigration of cells*, *Hyperplasia or Transformation of fibroblast cell lines* (Fig. 2), and to protein and muscle organization and accretion such as *Mass of organism*, *Quantity of protein in blood* and *Organization of cytoskeleton* (Fig. 3). Consistently, DE genes in IBxDU pigs clustered in 3 gene networks related to *Cell-To-Cell Signaling and Interaction*, *Hematological System Development and Function*, *Immune Cell Trafficking, Post-Translational Modification*, *Protein Folding*, *Cellular Assembly and Organization*, *Cellular Movement*, *Cell Signaling* and *Cellular Function and Maintenance.*

Fig 2: Enriched biological functions related to cell growth in IBxDU pigs.

The network generated by IPA software shows enriched biological functions in IBxDU pigs (orange color) and genes predicted to be involved in enrichment of these functions. Genes upregulated in IB pigs are highlighted in green color and genes upregulated in IBxDU pigs are highlighted in red color. Lines ending in arrow represent activation; lines ending in a bar represent inhibition. Orange lines indicate activation of the biological function. Yellow lines represent findings inconsistent with the state of the biological function and grey lines a non predicted effect.

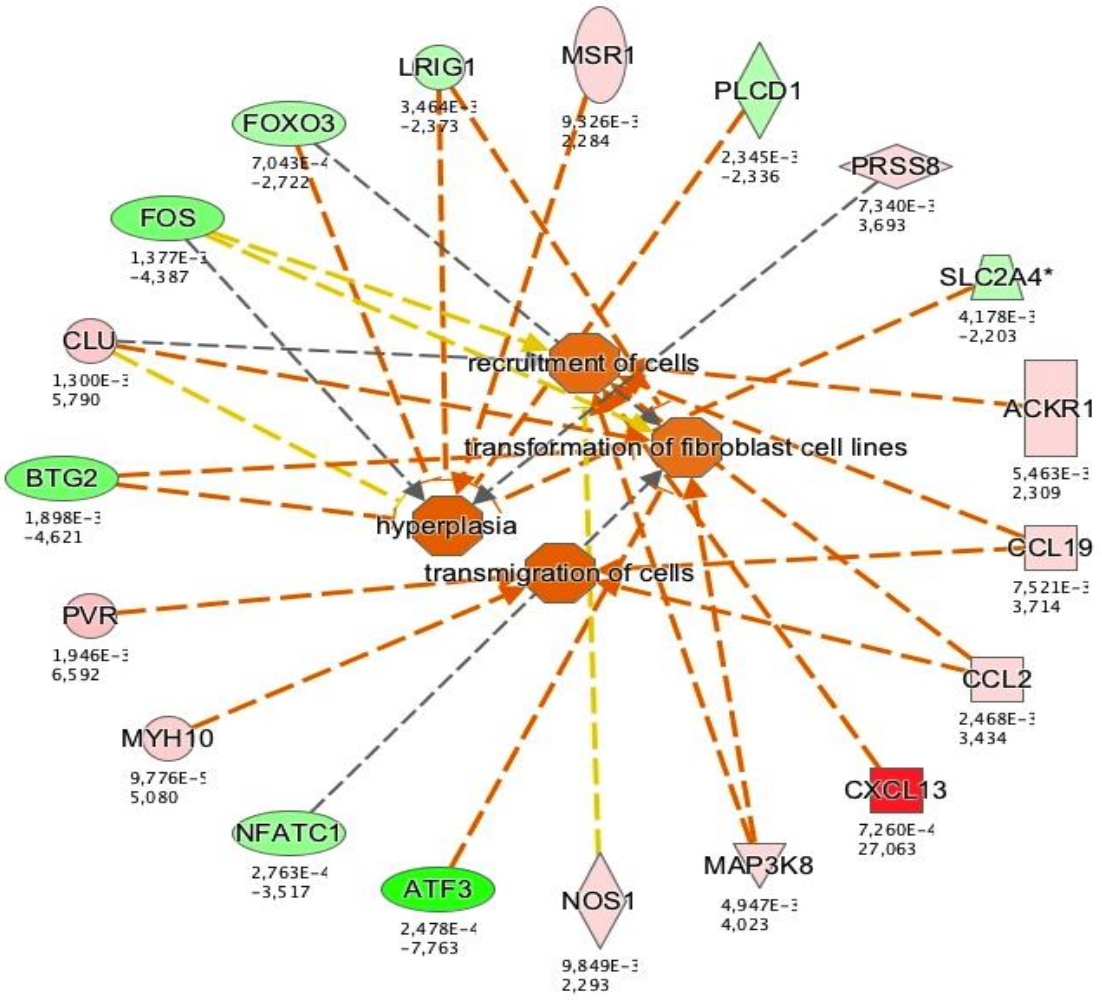
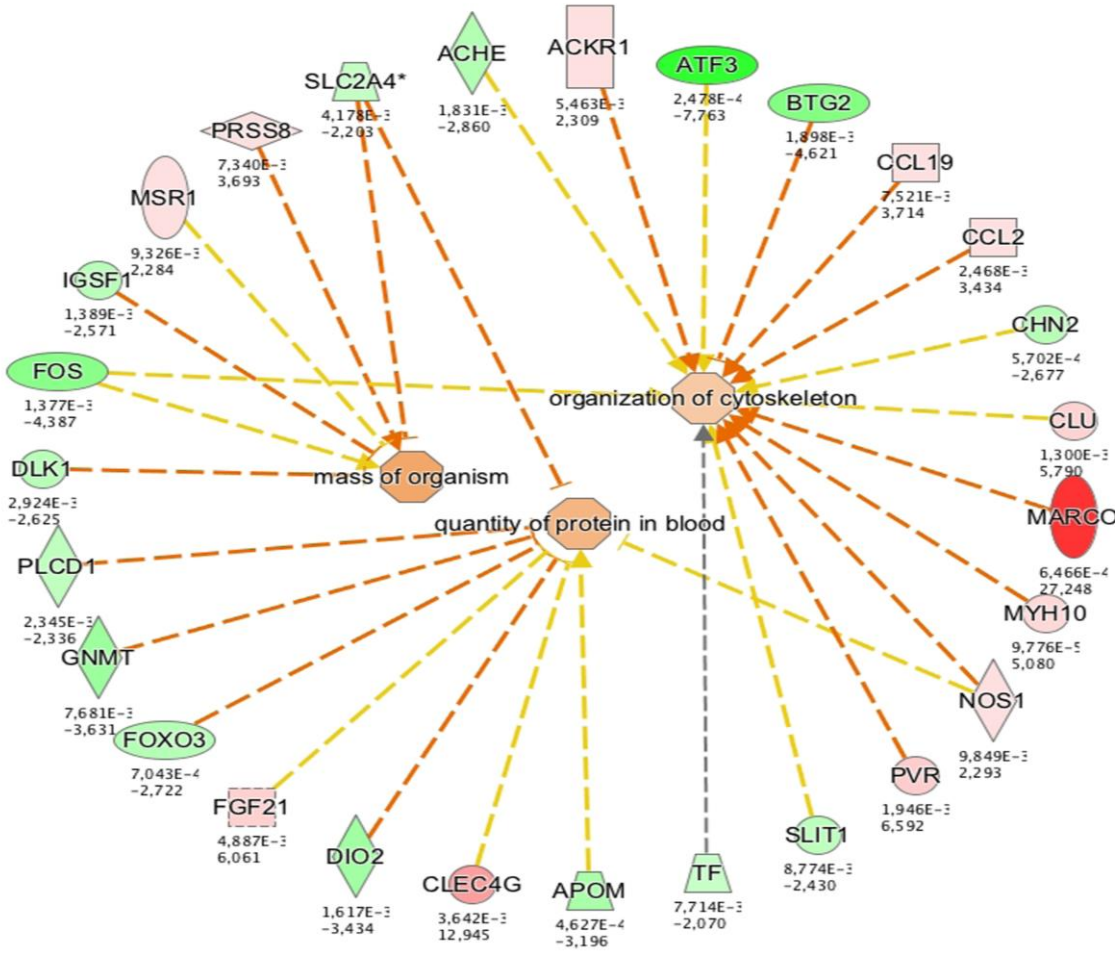


Fig 3: Enriched biological functions potentially related to muscle growth in IBxDU pigs.

The network generated by IPA software shows enriched biological functions in IBxDU pigs (orange color) and genes predicted to be involved in enrichment of these functions. Genes upregulated in IB pigs are highlighted in green color and genes upregulated in IBxDU pigs are highlighted in red color. Lines ending in arrow represent activation; lines ending in a bar represent inhibition. Orange lines indicate activation of the biological function. Yellow lines represent findings inconsistent with the state of the biological function and grey lines a non predicted effect.



Some IBxDU upregulated genes involved in those functions were chemokines (i.e. *CCL19, CCL2* or *CXCL13*) and chemokine receptors (*ACKR1*), which have been reported to be involved in the process of myogenesis and muscle regeneration (Iwasaki et al., 2013). In our study, chemokines were associated with functions such as transmigration and recruitment of cells and organization of cytoskeleton (Figs. 2 and 3), which could be related to muscle cell growth. Moreover, *MYH10*, a non-muscle myosin that regulates actin cytoskeleton remodeling, was found overexpressed in IBxDU and involved in cytoskeleton reorganization, a critical process during myogenesis (Posey et al., 2014). Consistently, the *Actin Cytoskeleton Signaling* pathway was also enriched in IBxDU pigs in both the present study and at 28 day of age (Ovilo et al., 2014b).

Mass of organism, associated with the overexpression of *PRSS8*, was predicted by IPA software to be enriched in IBxDU piglets, in accordance with the phenotypic differences in body weight and size found between pure and crossbred pigs. In agreement, a decrease in body weight was reported in mutant rats for gene *PRSS8* (Frateschi et al., 2012).

The enrichment of protein and muscle development-related pathways ($p < 0.05$), such as *Citrulline-Nitric Oxide Cycle*, *Superpathway of Citrulline Metabolism or nNOS Signaling in Skeletal Muscle*, support the greater muscle development in IBxDU piglets. Citrulline is a non-essential amino acid that plays a role in muscle development and affects body composition in rats, increasing lean and decreasing adipose tissue when it is provided in the diet (Moinard et al., 2015). The *nNOS Signaling in Skeletal Muscle Cells* modifies the blood flow to the muscle (Bredt, 2003). Thus, the activation of these pathways suggests a greater nutrient input to muscle tissues in those pigs.

Upregulated functions and pathways in *Biceps femoris* muscle from both Duroc-crossbred and purebred Iberian piglets

Development-related processes depend on the genetic background, but also on the growth stage. At birth, when growth is a very active process, several functions and pathways enriched in both genetic types were observed. However, these common processes ended up in different phenotypic consequences. This might be because developmental mechanisms such as muscle growth or immune system development are tightly regulated and the final output depends on both the balance of activating and inhibiting pathways and the expression levels of genes involved in such processes.

Cellular growth

The GO terms enrichment analysis retrieved several GO terms related to normal cell cycle and growth, such as cell differentiation, cellular protein metabolic process or macromolecule biosynthetic process that were common between both genetic types, supporting the importance of these processes in the early development of pigs. Accordingly, in 3 months-old Casertana pig (an autochthonous Italian fatty pig breed), GO terms close to the aforementioned were upregulated when compared to 6 months old pigs (D'Andrea et al., 2011).

Protein metabolism

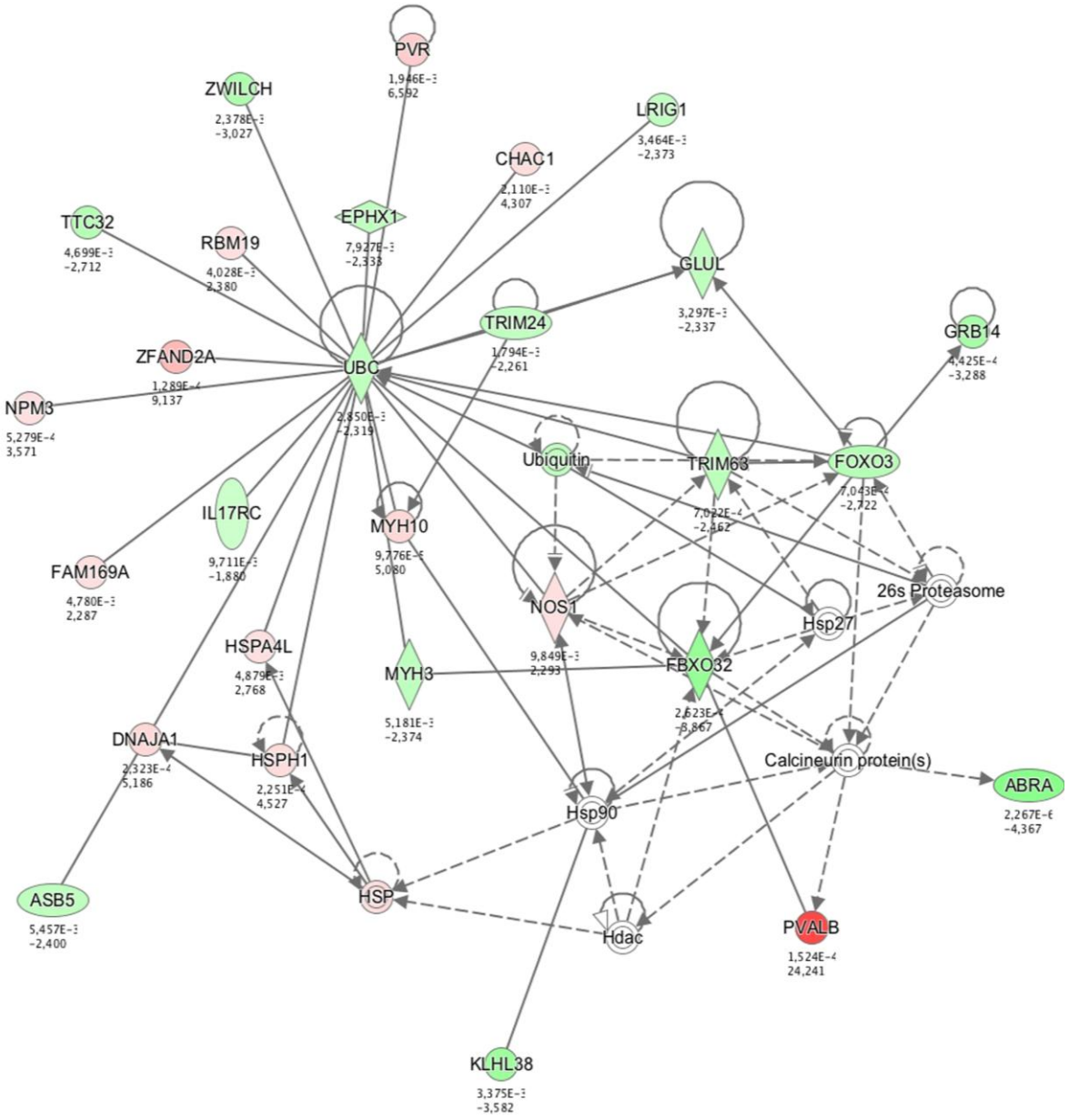
The importance of protein metabolism at birth is supported by the activation of the protein ubiquitination process in both genetic types. The *Protein Ubiquitination Pathway* was enriched in crossbred piglets ($p = 0.005$; Table 3), where mainly members of the family of Heat Shock Proteins (*HSPH1, HSPA4L* and *DNAJA1*) were found upregulated in IBxDU pigs. *DNAJA1*, together with *HSP27* have been reported to play a role in both cellular stress and meat quality; their expression was found to decrease IMF content (Kim et al., 2010), consistently with the results observed in IBxDU pigs. However, no enrichment in atrophy or degradation of muscle-related functions was observed

in IBxDU piglets. Conversely, IB muscle showed activation of functions related to muscle atrophy (Fig. 1), mainly driven by genes that stimulate muscle atrophy by means of the ubiquitin proteasome system (UPS) (Jaitovich et al., 2015). This system was also upregulated in IB when compared to IBxDU pigs at 28 days of age (Ovilo et al., 2014b); and in Basque (a French pig breed with low lean meat and high fat contents) when compared to Large White pigs (Damon et al., 2012). Supporting the evidence of an activated protein ubiquitination process, the gene coding for Ubiquitin C (*UBC*) and *FBXO32* (one of the three ligase enzymes, together with *TRIM63* of the UPS) were also overexpressed in IB pigs.

In accordance with these results, genes implicated in these processes were found in the most significant gene network involving DE genes in both genetic types. This network was associated with *Skeletal and Muscular Disorders*, as well as with *Cellular Compromise*, *Organismal Injury and Abnormalities* (Fig. 4) and showed several genes related to protein catabolism (*UBC, HSP, TRIM63* or *FOXO3*) among the most central genes in the network, which supports the importance of these genes in protein catabolism, a very active process in developing IBxDU and especially in IB pigs.

Fig 4: Gene network containing DE genes related to Cellular Compromise,Organismal Injury and Abnormalities and Skeletal and Muscular Disorders.

Genes upregulated in IB pigs are highlighted in green color and genes upregulated in IBxDU pigs are highlighted in red color. Genes colored in white are added by IPA software to complete the network.



Iberian pigs show low protein accretion potential, although higher relative protein synthesis rate has been reported in IB than in lean Landrace pigs (Rivera-Ferre et al., 2005). Since IB muscles are smaller than Landrace ones, it was suggested that a higher protein turnover rate in IB pigs should be responsible for the differences between protein synthesis and deposition (Rivera-Ferre et al., 2005). In this context, it seems evident that this increased protein turnover in IB pigs is related to the activated muscle atrophy-related genes found in this study. Moreover, Damon et al. (2012)

suggested that the activation of the UPS might affect meat tenderness, since proteasome would be one of the main endogenous proteolityc systems contributing post-mortem meat tenderization.

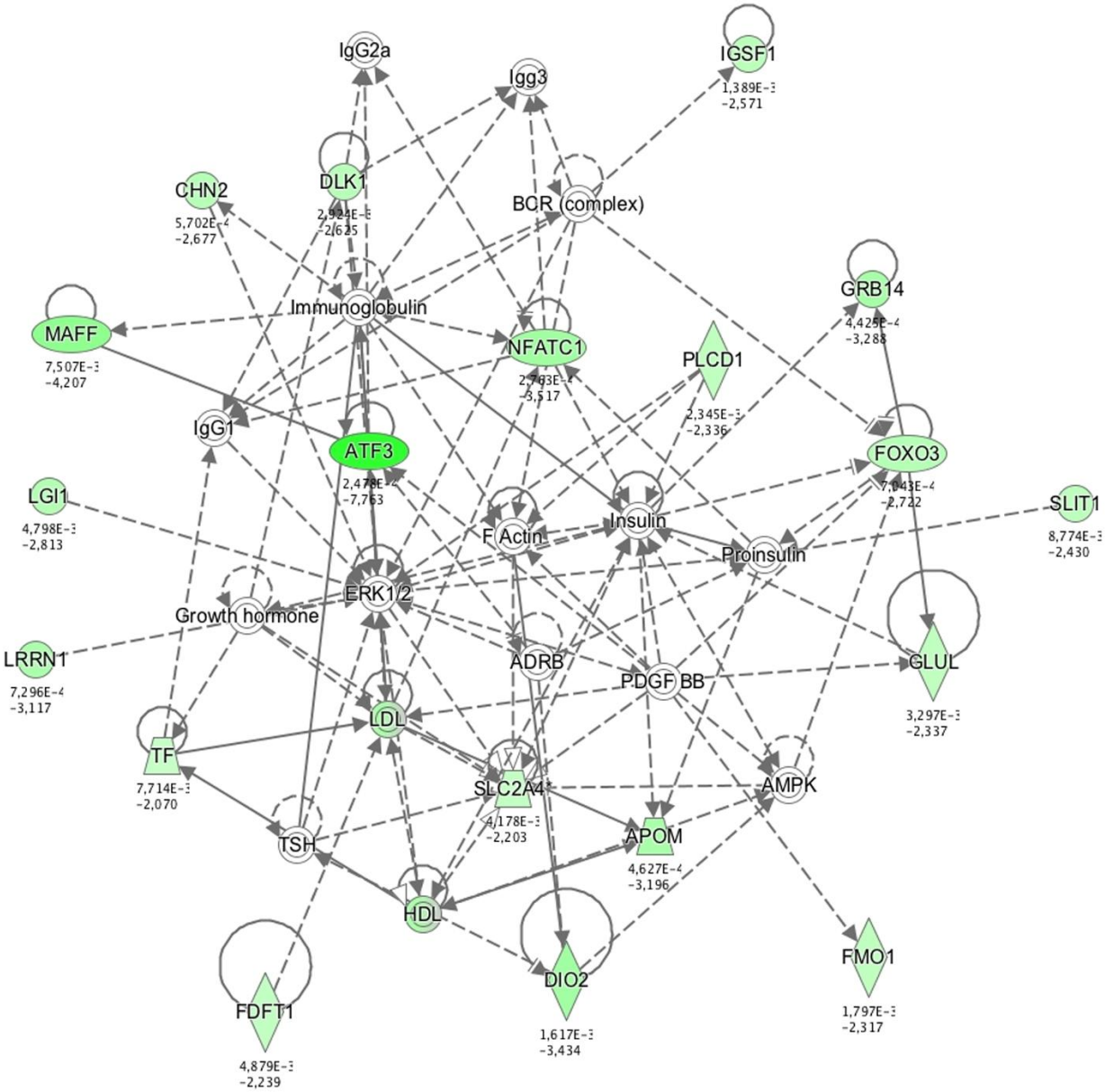
The UPS is also interconnected to the unfolded protein response (UPR), activated under endoplasmic reticulum stress situations. The UPR is involved in degradation of un and misfolded proteins and recently associated to the control of adipogenesis under situations of endoplasmic reticulum stress (Zheng et al., 2010; Koc et al., 2015)]. It is noteworthy that *ATF3*, a transcription factor inducible by endoplasmic reticulum stress (Hai and Hartman, 2001) was upregulated in IB pigs. This could suggest certain level of endoplasmic reticulum stress in IB pigs that might be related to preadipocyte differentiation.

Cholesterol metabolism

The importance of cholesterol in early stages of development could be the cause for the activation of pathways related to cholesterol metabolism and biosynthesis in both genetic types. For example, the *LXR/RXR activation pathway*, involved in the regulation of lipid metabolism, inflammation and cholesterol to bile acid catabolism, was enriched in both IB and IBxDU newborns ($p = 0.006$ and $p < 0.001$, respectively). On the contrary, at 28 days of age, this pathway was enriched in IB pigs (Ovilo et al., 2014b). The *atherosclerosis signaling pathway* (enriched in IBxDU pigs) or the *Cholesterol Biosynthesis* and the *Epoxysqualene Biosynthesis pathways* (enriched in IB pigs) were also identified as relevant pathways. However, phenotypic observations reflect a more active cholesterol and triglycerides biosynthesis in IB than in IBxDU piglets (Table 1). This could be due to the upregulation of different genes in each genetic type: upregulated genes in IB pigs associated with these pathways were closely related to the cholesterol and lipid metabolism (*FDFT, TF* and *APOM*). Accordingly, genes related to cholesterol and lipid metabolism such as *RXRG, USF1, LPL* or some apolipoproteins have been proposed as candidate genes involved in the familial combined hyperlipidemia in human (Pajukanta et al., 2004; Pushpakom et al., 2013; Luo et al., 2015), characterized by high levels of plasma cholesterol and triglycerides, as observed in IB pigs. Moreover, the aforementioned upregulated genes in IB pigs were connected in a network associated with functions such as *Amino Acid Metabolism*, *Molecular Transport* and *Small Molecule Biochemistry* (Fig. 5). On the other hand, genes involved in such cholesterol-related pathways that were upregulated in IBxDU piglets where associated to the immune response (*MSR1* and *CCL2*).

Fig 5: Gene network containing genes upregulated in IB pigs related to *Amino Acid Metabolism*, *Molecular Transport* and *Small Molecule Biochemistry*.

Genes upregulated in IB pigs are highlighted in green color and genes upregulated in IBxDU pigs are highlighted in red color. Genes colored in white are added by IPA software to complete the network.



Immune response

The immune system is not fully developed at birth in pigs (Becker and Misfeldt, 1993), and thus, related pathways and functions were upregulated in both in IB and IBxDU newborns. Some of these pathways include *PI3K Signaling in B Lymphocytes, April Mediated Signaling* or *B Cell Activating Factor Signaling* (enriched in IB pigs) and *Agranulocyte Adhesion and Diapedesis* or *IL-12 Signaling and Production in Macrophages* (enriched in IBxDU pigs). Moreover, the pathway *IL-17A Signaling in Fibroblasts* was enriched in both genetic types. On the other hand, the GO term *Positive regulation*

of immune system process was enriched in IBxDU but not in IB piglets. This is consistent with the aforementioned upregulation of genes such as *MARCO* and *CXCL13* in Duroc-crossbred pigs, suggesting a more developed immune system in IBxDU than IB newborns. It has been previously reported that the immune system is affected by pig breed (Sutherland et al., 2005) and domestication (Bergman et al., 2010). An overrepresentation of genes related to the immune system was reported in wild boars when compared to domestic pig breeds (Amaral et al., 2011). However, to our knowledge there are no previous studies assessing differences in immune efficiency introduced by sire line.

Immune system-related genes are also involved in numerous other biological processes, such as fat accumulation, closely associated with inflammation (Balasubramanyam, 2013; Exley et al., 2014). Thus, the enrichment of pathways related to the immune system could be related to lipid accumulation, although it has also been related to lower backfat thickness in pigs (Xing et al., 2015). It is noteworthy that no biological functions related to the immune system were found enriched in IB pigs, which might suggest that upregulation of immune system-related pathways might be related to adipose tissue development rather than to the immune system development in IB pigs.

The reported active biological functions in IB and IBxDU pigs suggest a different predisposition for cell and tissue growth between genetic types, being IB pig metabolism intended to energy storage and IBxDU pig metabolism to cell growth and differentiation. These differences in biological regulation are in agreement with phenotypic differences found between the two genetic types.

Regulatory transcription factors

We performed a regulatory factors study to investigate the driving molecular mechanisms responsible for the differences in gene expression observed between genetic types.

A total of 723 TRF, obtained from the animal TFDB, showed expression values above 0.5 FPKM in our pigs and were thus considered as expressing TRF. Among them, 92 TRF potentially affected the gene expression profile in IB and IBxDU muscles (Table 4). We considered TRF that were either DE (7 TRF), identified by IPA software as regulators (45 TRF) or identified in the RIFs study (48 TRF) (Table 4).

Table 4: Potential regulators affecting gene expression that are: a)differentially expressed (DE) between IB and IBxDU,b) identified by Ingenuity Pathways Analysis (IPA) software or c) identified by RIFs study.

Ensembl ID	GENE	DE regulators		IPA - Regulators		RIFs study	
		p-value	FC	Z-score	*p*-value	RIF1	RIF2
ENSSSCG00000006036	*ABRA*	2.27E-06	-4.37				
ENSSSCG00000008123	*ARID5A*						2.01
ENSSSCG00000015972	*ATF2*				4.40E-02		
ENSSSCG00000015595	*ATF3*	2.48E-04	-7.76				
ENSSSCG00000000084	*ATF4*				1.42E-02		
ENSSSCG00000012241	*BCOR*				4.17E-03		
ENSSSCG00000013397	*BMAL1*						-2.22
ENSSSCG00000008377	*CCT4*						-2.11
ENSSSCG00000002866	*CEBPA*			-2.42	7.32E-03		
ENSSSCG00000002867	*CEBPG*					3.60	
ENSSSCG00000006752	*CSDE1*					2.45	
ENSSSCG00000011274	*CTNNB1*			-2.18	1.01E-02		
ENSSSCG00000014336	*EGR1*			-0.25	5.28E-03		
ENSSSCG00000010224	*EGR2*				2.01E-02		2.21
ENSSSCG00000008443	*EPAS1*				4.01E-02		
ENSSSCG00000002383	*FOS*	1.38E-03	-4.39	0.38	6.14E-03		
ENSSSCG00000012967	*FOSL1*				2.76E-02		
ENSSSCG00000007576	*FOXK1*					2.35	
ENSSSCG00000009370	*FOXO1*				1.03E-02	3.22	
ENSSSCG00000004387	*FOXO3*	7.04E-04	-2.72	-2.36	5.05E-06		
ENSSSCG00000001619	*FOXP4*					2.79	
ENSSSCG00000015733	*GLI2*					2.60	
ENSSSCG00000007720	*GTF2IRD1*				1.66E-02		
ENSSSCG00000000846	*HCFC2*					2.34	
ENSSSCG00000014388	*HDAC3*				2.36E-02		
ENSSSCG00000010472	*HHEX*				4.89E-02		
ENSSSCG00000004138	*HIVEP2*				1.70E-04		
ENSSSCG00000009327	*HMGB1*				3.67E-03		
ENSSSCG00000009704	*HMGB2*					2.55	
ENSSSCG00000008898	*HOPX*				1.66E-02		
ENSSSCG00000015985	*HOXD3*					3.59	
ENSSSCG00000005917	*HSF1*				9.28E-05		
ENSSSCG00000004238	*HSF2*				6.06E-03		
ENSSSCG00000014277	*IRF1*				2.49E-02		2.19
ENSSSCG00000003178	*IRF3*				3.13E-02		
ENSSSCG00000012853	*IRF7*				3.08E-02		
ENSSSCG00000008119	*KCNIP3*					2.34	
ENSSSCG00000010928	*KDM5B*						-2.10
ENSSSCG00000006928	*LMO4*					2.64	
ENSSSCG00000004528	*MBD2*				2.44E-02		
ENSSSCG00000000552	*MED21*					2.35	
ENSSSCG00000005720	*MED27*					2.18	
ENSSSCG00000006482	*MEF2D*				6.93E-03		
ENSSSCG00000012534	*MORF4L2*						-2.11
ENSSSCG00000013507	*MPND*						2.18
ENSSSCG00000012114	*MSL3*						2.34
ENSSSCG00000017882	*MYBBP1A*				2.07E-02		
ENSSSCG00000013375	*MYOD1*				7.26E-03		

Ensembl ID	GEN	DE regulators		IPA - Regulators		RIFs study	
		p-value	FC	Zscore	*p*-value	RIF1	RIF2
ENSSSCG00000015475	*MYOG*				8.17E-06		
ENSSSCG00000010399	*NCOA4*					3.59	
ENSSSCG00000015987	*NFE2L2*			-2.20	2.44E-02		
ENSSSCG00000001952	*NFKBIA*			-1.27	3.40E-02		
ENSSSCG00000001703	*NFKBIE*				4.09E-02		
ENSSSCG00000005385	*NOR-1*	8.17E-04	-6.56				
ENSSSCG00000009856	*NOS1*	9.85E-03	2.29		3.78E-05		
ENSSSCG00000006689	*PIAS3*				3.69E-02		
ENSSSCG00000014437	*PPARGC1B*				1.65E-02		
ENSSSCG00000009746	*RAN*						-2.25
ENSSSCG00000009401	*RB1*			1.17	2.29E-02		
ENSSSCG00000008388	*REL*				2.04E-03		
ENSSSCG00000012981	*RELA*			0.75	6.16E-04		
ENSSSCG00000017071	*SAP30L*						-2.28
ENSSSCG00000011201	*SATB1*						-2.32
ENSSSCG00000001880	*SIN3A*						-2.12
ENSSSCG00000004952	*SMAD2*						2.43
ENSSSCG00000004524	*SMAD4*				4.94E-02		
ENSSSCG00000005232	*SMARCA2*						-2.47
ENSSSCG00000013629	*SMARCA4*				4.00E-02		
ENSSSCG00000006256	*SOX17*				3.29E-02		
ENSSSCG00000017403	*STAT3*				3.52E-02		
ENSSSCG00000017406	*STAT5B*					2.22	
ENSSSCG00000010638	*TCF7L2*				2.68E-02		
ENSSSCG00000001092	*TDP2*					2.44	
ENSSSCG00000001544	*TEAD3*						-2.06
ENSSSCG00000017950	*TP53*			-1.44	1.21E-04	3.48	1.69
ENSSSCG00000007208	*TRIB3*					2.84	
ENSSSCG00000027684	*TRIM63*	7.02E-04	-2.46		1.25E-02		
ENSSSCG00000015370	*TWIST1*				3.28E-03		
ENSSSCG00000006790	*WDR77*						-2.18
ENSSSCG00000011953	*ZBTB11*					2.10	
ENSSSCG00000007958	*ZNF174*					2.31	
ENSSSCG00000003233	*ZNF175*					2.86	
ENSSSCG00000002838	*ZNF423*					2.26	
ENSSSCG00000003070	*ZNF45*					2.24	
ENSSSCG00000007767	*ZNF668*						2.26
ENSSSCG00000001206	*ZSCAN26*						-2.84
ENSSSCG00000002877	*ZNF181*					2.50	
ENSSSCG00000003244							2.05
ENSSSCG00000011620							-2.41
ENSSSCG00000011943							-2.07
ENSSSCG00000013384							1.96
ENSSSCG00000016700						3.43	

FC: Fold-Change

Z: Z-score: reflects the activation state of predicted transcriptional regulators. It is based on the experimentally observed gene expression, and on literature-derived regulation direction information, which can be either "activating" or "inhibiting"

RIF1 (z)extreme scores identify those transcription factors that are consistently most differentially co-expressed with highly abundant and highly DE genes. Bootstrap 95% and 99% confidence intervals for RIF1 z-scores: -1.996/2.074 and -2.883/2.918, respectively.

RIF2 (z)extreme scores identify transcription factors with the most altered ability to predict the abundance of DE genes. Bootstrap 95% and 99% confidence intervals for RIF2 z-scores: -2.036/1.953 and -2.609/2.490, respectively

In the present study, we found 7 TRF showing differences in expression level between genetic types. Most of them (*ABRA, ATF3, FOS, FOXO3, NOR1, TRIM63*) were upregulated in IB. These genes are related to muscle and adipose tissue development (Fig. 1) and most of them have been mentioned in the previous section, but some of them may be highlighted and deserve additional comments. For example, one of the genes with greater expression differences (*ATF3*) was also an identified regulator in the *Animal TFDB*, suggesting an important role in the gene expression differences observed. *NOR1* showed also an important expression difference (6.56x) and codes for a nuclear receptor involved in a wide array of functions such as inflammation, cell cycle regulation, apoptosis, steroidogenesis, adipogenesis, angiogenesis and energy metabolism. Moreover, it has been found overexpressed in obese when compared to normal humans and its expression went back to normal values after fat loss (Veum et al., 2012). These findings are in accordance with the results of the present study, where animals with higher IMF content showed greater muscular expression of *NOR1*.

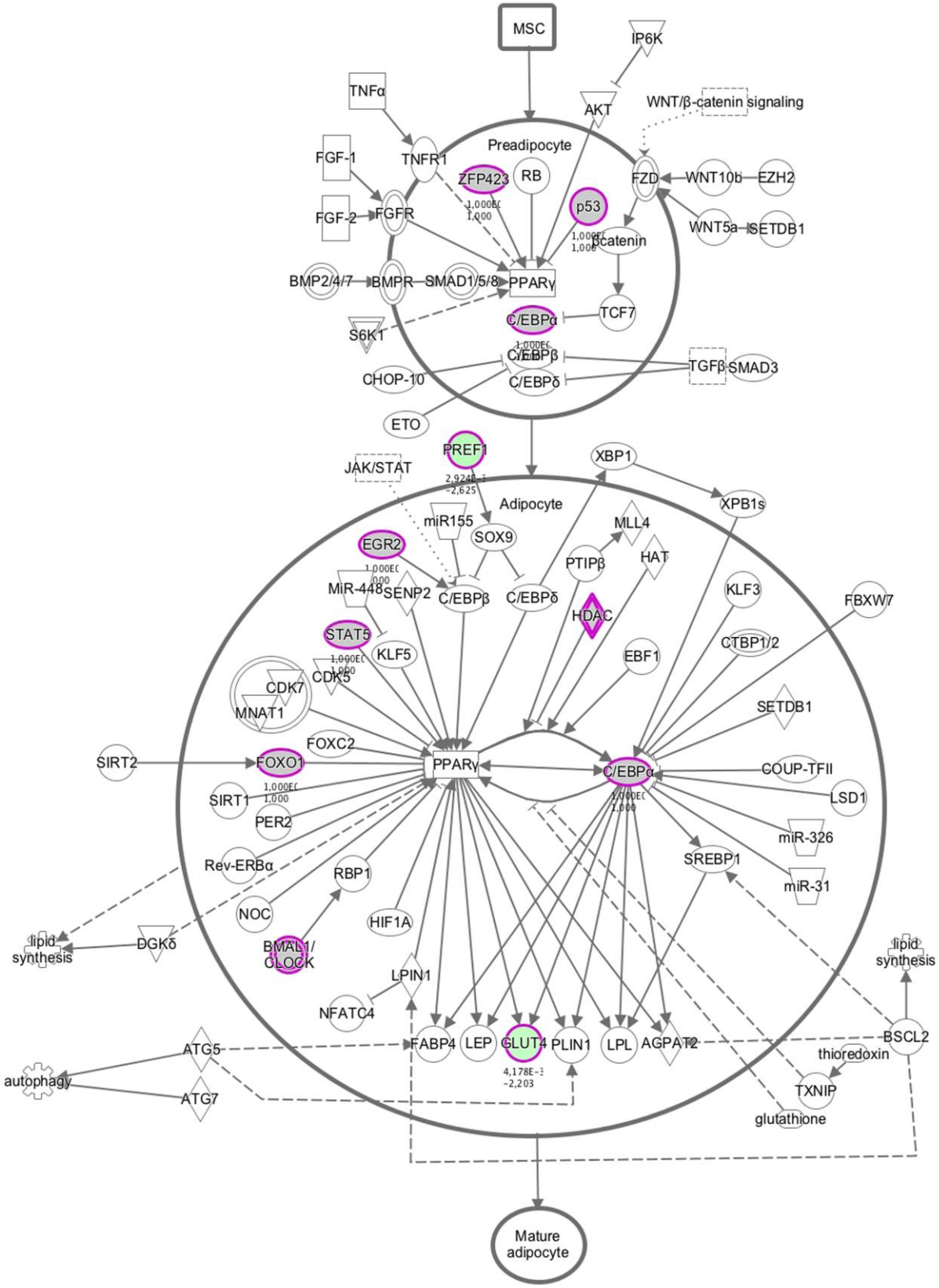
IPA software is a potent tool to identify regulators based on previous knowledge and bibliographic references. On the other hand, the RIFs analysis, based on co-expression information in our dataset, complements the bibliographic approach (IPA). The combination of these two methods is a powerful strategy to identify TRF. In the present work, IPA software identified 45 TRF affecting the DE genes, while the RIFs metrics identified 48. Four TRFs were identified following the two approaches (*EGR2, FOXO1, IRF1* and *TP53*). *EGR2* is a growth factor that promotes adipocyte differentiation (Boyle et al., 2009), while TP53 has been reported to affect cell metabolism (Vousden and Ryan, 2009; Berkers et al., 2013). FOXO1 is a member of the forkhead family of TRF, which exerts important regulatory functions in developmental processes including muscle development (Wijchers et al., 2006; Hannenhalli and Kaestner, 2009). *FOXO1* regulates expression of several adipogenic genes, including *PPARG* (Gupta et al., 2013) and muscle cells differentiation in association with *SMAD* (Allen and Unterman, 2007), although inconsistent information exits regarding its specific role (Allen and Unterman, 2007; Hakuno et al., 2011). Moreover, several TRF with known functions on adipogenesis and lipid metabolism were identified in the present regulators analysis (*CEBPA, CEBPG*, *ZFP423EGR1, ATF2, ATF4* and *PPARGC1B)*. *PPARGC1B* is involved in fat oxidation, non-oxidative glucose metabolism, mitochondrial biogenesis and the regulation of the energy expenditure (Handschin and Spiegelman, 2006) and was also identified as a potential regulator of gene expression differences at 28 days of age (Ovilo et al., 2014b). The identification of these genes as regulators in the present study suggests its implications in adipogenesis and in the observed fatness differences associated to sire breed.

Regulation of myogenesis is also a very complex process and several TRF involved on it have been identified (*FOXK1*, *FOXO4, MEF2D, MYOD1, HDAC3* and *MYOG)*. These genes play a central role in myogenesis regulation, acting sequentially in a signaling chain (Edmondson et al., 1992; Nabeshima et al., 1993; Ivana et al., 2005; Zhao et al., 2011; Shi et al., 2012; Demmerle et al., 2013). Due to the importance of muscle development in the pork industry, several studies have assessed the differential expression of myogenic regulatory factors between breeds with different muscle growth potential (D'Andrea et al., 2011; Zhao et al., 2011; Ghosh et al., 2015; Zhao et al., 2015). The identification of these myogenic regulators as TRF in the present study, suggests that myogenesis is also activated immediately after birth, although it has been reported that expression significantly decreases in pigs after 90 days post-conception (Zhao et al., 2015). However, muscle development timing remains unclear, since it has also been hypothesized that muscle is still under development in 3 months-old Casertana pigs (D'Andrea et al., 2011). Moreover, genes involved in myogenesis inhibition (i.e. *SMAD* and *TWIST1*) were also identified as TRF affecting gene expression in IB and IBxDU pigs. The presence of both myogenesis activating and inhibiting genes reflects the complex regulation of this process in newborn piglets.

Muscle deposition not only depends on myogenesis; regulation of process such as angiogenesis, or protein degradation is also decisive in final muscle deposition. SOX17 and the heat shock transcription factor family members HSF1 and HSF2, involved in such processes (Yasuhara et al., 2011; Nishizawa et al., 2013; Lee et al., 2014), were identified in this TRF study.

To better understand interactions between the identified DE genes and TRF, a pathways enrichment analysis was performed combining information obtained in both analyses. The adipogenesis pathway was the most enriched pathway (S5 Table), accordingly with the differences in fatness observed in *BF* muscle of IB and IBxDU piglets. As observed in Fig. 6, the adipogenesis pathway includes several TRF (*ZFP423, P53, FOXO1, STAT5* or *EGR2*) and DE genes (*SLC4A2/GLUT4* and *DLK1*). It is noteworthy that *PPARG*, considered as the master regulator of adipogenesis, was not found to be DE or to regulate gene expression in newborn IB and IBxDU pigs. However, 6 out of the 8 identified TRFs involved in this pathway, regulated directly or indirectly the expression of *PPARG*.

Fig 6: Adipogenesis pathway.
Genes upregulated in IB pigs are highlighted in green color by IPA software. Genes colored in grey are transcription factors.



Structural analysis from RNA-Seq sequence data

A total of 433,667 putative variants were identified in IB and 461,438 in IBxDU pigs.

The total numbers of polymorphisms meeting the filtering criteria, and the location, frequency, aminoacid change and variant type distribution in both genetic types are shown in Table 5.

Both genetic types showed a similar variant number, with similar location and variant type distributions. Regarding variant frequency distribution, IB group showed slightly higher number of potentially fixed variants (frequency equal or higher than 90%) while crossbreds showed higher number of segregating variants (frequency lower than 90%), in agreement with the expectations for the comparison of one pure line with high inbreeding level as the Iberian pig (Esteve-Codina et al., 2011) and an F1 cross. Nevertheless, frequency estimations have to be considered with caution due to the limited sample size. Variants at different allelic frequencies in both genetic types were of special interest, especially those potentially fixed in IB and segregating in IBxDU (7,741 variants). Moreover, variations in protein-coding regions may give rise to non-synonymous changes in the amino acid sequence of the encoded protein and thus, are more likely to affect the protein structure and function (Uzun et al., 2007) probably leading to critical effects on a phenotype of interest (Fan et al., 2010). Out of the 7,741 potentially relevant variants, 846 produced a non-synonymous change.

Table 5. **Number of sequence variants present in Iberian (IB) and Duroc-crossbred (IBxDU) pigs RNA-Seq data and its distribution according to localization**, **frequency and polymorphism type**.

	IB	IBxDU
Total number of variants (after filtering)	120,998	125,382
Variant localization		
UTR Region	20,683	21,808
Coding region	100,315	103,574
Potentially fixed and segregating variants		
Fixed (G≥90%)	46336	40454
Segregate (G<90%)	74662	84928
AA change	18092	18556
Variant type		
SNV	88,415	92,416
MNV	11,842	11,873
Insertion	7,863	7,987
Deletion	9,539	9,699
Replacement	3,339	3,407

Filtering: Variants with coverage < 30 or frequency < 5% were dismissed
UTR Region: Untranslated region
AA change: Variants causing a aminoacid change in the protein
SNV: Single nucleotide variant
MNV: Multiple nucleotide variant
Replacement: Neighboring SNVs and insertions or deletions.

One of the goals of the present study was to identify structural variants in candidate genes involved in differences between IB and IBxDU piglets. Candidate genes were selected based on the following criteria: TRF simultaneously identified following IPA and RIF approaches (*EGR2, FOS, FOXO1, FOXO3, IRF1, NOS1, TRIM63, ABRA, ATF3* and *TP53*) and those coinciding with TRF previously identified in an IB and IBxDU piglets transcriptome comparison at 28 days of age (*HOXA9, STAT5B, ATF4* and *PPARGC1B*)

In order to identify structural variants in these strong candidate genes, we looked for differentially segregating SNPs in IB and IBxDU pigs. The total variant number in the whole set of selected genes was slightly larger in IBxDU than in IB pigs (365 and 294, respectively). Out of the identified variants, 177 were present in both genetic types, 117 were present uniquely in IB and 188 in IBxDU piglets. The supplementary file 6 (S6 Table) shows all the variants found in the 14 genes of interest. The genes *ATF4* and *HOXA9* did not show any polymorphism. The genes *ATF3, STAT5* and *ABRA* showed common polymorphisms between the two genetic types that were fixed or in high allelic frequency in IB pigs. Among them, *ABRA* presented 11 variants fixed in IB that segregate in IBxDU. None of these polymorphisms led to aminoacidic changes, nevertheless they might affect mRNA processing (splicing, maturation, stability, transport) and translation and thus, lead to altered mRNA folding and even expression (Johnson et al., 2008). Moreover, the 11 variants were found in cosegregation, which might lead to relevant changes in the mRNA. The identified forkhead family TRFs (*FOXO1* and *FOXO3A*) showed fixed and high frequency variants uniquely present in IBxDU pigs. Nevertheless, we identified a low frequency variant in IB pigs that produced a non-synonymous amino acid change in *FOXO1* gene. *NOS1* and *TP53* genes presented several variants in both genetic types. Variants in *NOS1* gene were mainly associated to the IBxDU type, two of them causing amino acid changes. On the other hand, variations in *TP53* were observed generally in IBxDU pigs. Considered as a tumor suppression gene, TP53 protein responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. It has been recently reported as a novel candidate for muscle development in pigs (Verardo et al., 2013) and was also involved in the adipogenesis pathway (Fig. 6). The *PPARGC1B* and TRIM63 genes were the most polymorphic ones among the studied genes (89 and 94 variants, respectively). Some of the variants found in this gene were associated to amino acid change. One of the variants identified in the *PPARGC1B* gene, at medium frequency in IB and absent in IBxDU, was predicted by SIFT to produce a deleterious amino acid change. Also a GCA-deletion, fixed in IB and segregating in IBxDU pigs, produces the lack of an alanine residue in the coded PPARGC1B protein. On the other hand, 15 variants raised missense amino acid changes and 7 produced frameshift alteration in the TRIM63 gene, which maintains muscle protein homeostasis by tagging the sarcomere proteins with ubiquitin for subsequent degradation by the UPS (Chen et al., 2012). Moreover the polymorphism

ENSSSCT00000028044:c.163G>C was predicted to be deleterious by SIFT metrics. Thus, these changes might potentially cause altered function in the proteins. Previous studies associated polymorphisms in *PPARGC1B* gene with type 2 diabetes (Villegas et al., 2014) and subcutaneous adiposity (Franks et al., 2014) in human. Moreover, *PPARGC1A* codes for a homologous protein of *PPARGC1B* and has been associated in pigs to carcass composition traits such as leaf fat weight, backfat thickness, and belly weight in a Meishan cross population (Jacobs et al., 2006).

Variants showing different segregation between genetic types may be associated with functional genetic differences, which potentially could affect gene regulation and metabolism. Specially, the polymorphisms associated to aminoacid changes detected in *FOXO1, NOR-1* and *PPARGC1B* genes might be candidate polymorphisms to partially explain the differential muscle and adipose growth between IB and IBxDU.

3.3.4- Conclusions

In the present study, differences in phenotype, transcriptome, metabolic pathways and transcriptional regulation were found between BF muscles from purebred and Duroc-crossbred Iberian newborn pigs. Phenotypic differences regarding body size, plasma cholesterol levels and IMF content were remarkable, even at this early age. This is concordant with the transcriptomic study, which revealed several DE genes related to adipose and muscle tissues development such as *DLK1, FGF21* and *UBC* genes. The interpretation of these results pointed out a differential regulation of several biological processes. For example, lipid metabolism and muscle atrophy were upregulated in IB pigs, in accordance with the greater adipose accretion and lower muscle growth observed in Iberian pig breed. However, muscle growth was upregulated in IBxDU pigs, characterized for a higher muscle development than IB pigs. These processes are closely related to meat quality and production traits. Protein catabolism and cholesterol metabolism were enriched in both genetic types, although phenotypic differences in plasma cholesterol suggest greater activation of this process in IB pigs. These results contribute to the understanding of molecular mechanisms driving phenotypic differences observed in IB and IBxDU pigs. Moreover, findings regarding lipid metabolism regulation might be of interest in research related to metabolic alterations in other species such as humans.

Also, we identified TRF that potentially regulate the observed transcriptomic differences. The different employed approaches allow highlighting several TRF especially interesting such as *CEBPs, EGRs, ATFs, PPARGC1B, FOXOs, TRIM63, MEFD2D, MYOD1* or *MYOG*.

Finally, genetic structural variations that might be associated to changes in expression and protein function were identified. Differentially segregating SNPs in IB and IBxDU piglets associated to those TRF that were more consistently identified were of special interest. Among them, PPARGC1B and *TRIM63* showed non-synonymous variants that might differentially regulate their function in IB and

IBxDU pigs. Taken together, results found in the present study provide information about candidate genes and genetic polymorphisms potentially involved in phenotypic differences between IB and IBxDU associated to meat quality and production traits. Further validation of these genes and polymorphisms would contribute to their use in future breeding programs.

Acknowledgements

We are grateful to the staff of "S.A.T Vallehermoso" and to De la Torre, I. for technical support. The experimental work was supported by funds from the Spanish Ministry of Science and Innovation (project AGL2010-21991-C03) and the Ministry of Economy and Competitiveness (project AGL2013-48121-C3), co-funded by FEDER. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Miriam Ayuso is granted by the Ministry of Science and Innovation (BES-2011-045136).

Authors' contributions

Conceived and designed the experiment: CO, CLB, AGB; performed the experiment: MA, BIR, AIR, RB, YN, CB; analyzed data: MA, AF, AIF, CO, JFM, AC; contributed reagents/materials/analysis tools: AGB, BIR, AIR; wrote the manuscript: MA, CO. All authors read and approved the final version of the manuscript.

Supporting Information

S1 Table. Gene information, primer sequences, amplicon size and efficiency of genes selected for qPCR validation

S2 Table. Genes found differentially expressed between purebred (IB) and Duroc-crossbred (IBxDU) newborn pigs.

S3 Table. RNA-Seq and qPCR validation results and correlation coefficient (r) between the two used methodologies

S4 Table.Enriched biological functions identified by IPA software in purebred (IB) and Duroc-crossbred (IBxDU) Iberian pigs ($p<0.01$)

S5 Table. Canonical pathways enriched in IB and IBxDU animals based on DE genes and TFR identified either by IPA or by RIFs study

S6 Table. Structural variants identified in 14 candidate genes (*EGR2, FOS, FOXO1, FOXO3, IRF1, NOS1, TRIM63, ABRA, ATF3*, *TP53*, *HOXA9, STAT5B, ATF4* and *PPARGC1B).*

3.2 CAPITULO 2: La edad, el músculo y el tipo genético modifican el transcriptoma muscular en cerdos: efecto sobre la expression génica y factores reguladores involucrados en el crecimiento y el metabolismo.

Age, muscle and genetic type modify muscle transcriptome in pigs: effects on gene expression and regulatory factors involved in growth and metabolism.

Miriam Ayuso, Almudena Fernández, Yolanda Núñez, Rita Benítez, Beatriz Isabel, Carmen Barragán, Ana I. Fernández, Ana I. Rey, Juan F. Medrano, Ángela Cánovas, Antonio González-Bulnes, Clemente J. López-Bote, Cristina Óvilo.

2015. En preparación.

Age, muscle and genetic type modify muscle transcriptome in pigs: effects on gene expression and regulatory factors involved in growth and metabolism.

Lomgissimus dorsi muscle transcriptome in heavy pigs

Miriam Ayuso[1], Almudena Fernández[2], Yolanda Núñez[2], Rita Benítez[2], Beatriz Isabel[1], Carmen Barragán[2], Ana I. Fernández[2], Ana I. Rey[1], Juan F. Medrano[4], Ángela Cánovas[4¥], Antonio González-Bulnes[3], Clemente J. López-Bote[1], Cristina Óvilo[2*].

[1]Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense, Madrid, Spain

[2]Departamento de Mejora Genética Animal, INIA, Madrid, Spain

[3]Comparative Physiology Lab SGIT-INIA, 28040 Madrid, Spain

[4]Department of Animal Science, University of California Davis, Davis, California, United States of America

[¥]Current affiliation: Department of Animal and Poultry Science. University of Guelph, Guelph, Center for Genetic Improvement of Livestock, Ontario

*Corresponding author: ovilo@inia.es (CO)

Keywords: transcriptome; RNA-Seq; differential expression; Iberian pig; muscle; regulatory mechanisms; growth; lipid metabolism; fat deposition.

3.2.1- Abstract

Iberian pig production includes both purebred (IB) and Duroc-crossbred (IBxDU) pigs, which show important differences in growth, fattening and tissue composition. This experiment was conducted to investigate the effects of age, genetic type and muscle (*Longissimus dorsi* (LD) *vs Biceps femoris*) on gene expression, metabolic pathways and transcriptional regulation. Nine IB and 10 IBxDU piglets were slaughtered at birth, and 7 IB and 10 IBxDU were slaughtered at four months of age. Carcass traits were measured and samples from LD were taken to study intramuscular fat (IMF) content and composition and to analyze the muscle transcriptome with RNA-Seq technology. Previous phenotype and transcriptome data obtained from BF muscle of the same newborns was employed in the analyses. Carcasses were lighter and shorter in IB than in IBxDU neonates ($p < 0.001$) but not in four months old pigs. Similarly, higher cholesterol and triglycerides levels were observed in IB than IBxDU neonates, but these differences disappeared with time. At four months of age, IB pigs showed greater IMF content and lower ratio n6/n3 in LD ($p < 0.05$). Age significantly affected expression of 5,812 genes in the LD muscle transcriptome analysis ($p < 0.01$ and Fold change > 1.5), being 3,290 of them upregulated in neonates. However, muscle and genetic type effects were smaller. The expression of 135 genes was affected by muscle type, 261 by genetic type at birth and 113 at four months of age. Age was the main factor affecting phenotype and gene expression, newborn pigs transcriptome reflected a highly proliferative developmental stage, with upregulation of genes and enrichment of pathways and functions related to cellular growth and anabolic processes. Opposite results were observed in older pigs, in which upregulated genes were closely related to catabolism and muscle functioning. Genetic type phenotypic results and enrichment of pathways involved in muscle growth in newborn IBxDU pigs suggest a higher prenatal growth in these pigs. However, IB pigs showed enrichment of pathways and functions involved in protein deposition, cellular growth and body size at four months of age, in agreement with a potential catch-up growth experienced by IB pigs during the early postnatal period. Moreover, energy metabolism differed between genotypes, IB pigs showing enrichment of pathways related to glucose and lipid metabolism at both developmental stages, while a pathway associated with greater basal energy expenditure, was enriched in four months old IBxDU pigs, in agreement with differences in IMF observed in LD muscle between IB and IBxDU pigs. Muscle type also affected gene expression; LD muscle seems to have a more active muscular and cell growth, while BF muscle metabolism points towards lipid metabolism and fatness. Several regulators controlling transcriptome in both genotypes were identified across muscles and ages (*SIM1, PVALB, MEFs, TCF7L2* or *FOXO1*), evidences of being strong candidate genes driving expression and thus, phenotypic differences between IB and IBxDU pigs.

The present study increases the knowledge about age and tissue-specific genes and molecular mechanisms underlying phenotypic differences observed between purebred and Duroc-crossbred Iberian pigs and highlight some candidate genes implicated in these molecular mechanisms.

3.2.2- Introduction

Modern pig production is mostly based on extensively selected breeds, which show optimized productivity and efficiency (Chang et al., 2003). However, in the Mediterranean basin these commercial breeds coexist with local breeds, employed for the production of unique high-quality traditional pork products. These breeds, usually known as fatty-pigs, are smaller in size, have not undergone intense genetic selection and are less productive than modern breeds (Gonzalez-Añover et al., 2010). Moreover, due to the traditional rearing system, under extensive free-range conditions, they are exposed to harsh environments and seasonal variations in food availability (associated with the development of a thrifty genotype) (Astiz et al., 2014).

The Iberian pig is the most representative Mediterranean traditional breed, and it has an important commercial value based on high quality dry-cured products in terms of consumers' health and acceptance (López-Bote, 1998). Iberian pig shows special growth, fattening and meat characteristics, mainly as a consequence of its particular high fat deposition and desaturation potential, high food intake associated with leptin resistance (Ovilo et al., 2005; Muñoz et al., 2009) and the elevated age at which those pigs are slaughtered (Daza et al., 2007; Rodriguez-Sanchez et al., 2010). Iberian pigs are subject of long productive cycles (12-18 months), associated with the development of some of their meat characteristics. It has been reported that parameters such as intramuscular fat (IMF) content, fatty acids (FA) profile or redness influence meat quality and improve as pigs mature (Unruh et al., 1996; Čandek-Potokar et al., 1998; Lebret et al., 2001; Latorre et al., 2003b; Rodriguez-Sanchez et al., 2010).

Due to the low productivity of this breed and to the long time required to obtain Iberian products, Duroc breed has been introduced as terminal sire to improve reproductive and growth performances and primal cuts yield. However, the introduction of Duroc genetics is associated with a decrease in meat quality, mainly determined by a decrease in IMF and monounsaturated fatty acids (MUFA) contents (Ventanas et al., 2006).

Intramuscular fat content and fatty acid composition are main factors affecting meat quality and strongly depend on genetic type, diet, anatomical location and age (Sharma et al., 1987; Latorre et al., 2003b; Wood et al., 2008). Intramuscular fat content is determined both by number and size of adipocytes within muscle fibers. During prenatal development and immediately after birth, preadipocyte differentiation is a very active process that slows down with animal growth (Sepe et al., 2011). Later in growth hypertrophy is the most important issue affecting IMF content, although hyperplasia is maintained in the adult animal to a lesser extent (Gregoire et al., 1998). Hypertrophy is determined by fat (mainly triacylglycerides) accumulation in mature adipocytes and depends on lipogenesis and lipolysis. Therefore, changes in fatness-related processes are expected between newborn and juvenile pigs and sequential studies at different ages are required for understanding

the differences in molecular mechanisms driving fat accretion in Iberian pigs along growth. Moreover, fat accumulation also depends on the anatomical location. Several studies reported differences in lipid composition (probably associated with muscle fiber composition) (Sharma et al., 1987; Leseigneur-Meynier and Gandemer, 1991), oxidative properties and IMF content (Karlsson et al., 1993; Andrés et al., 2001) between muscles.

Due to the importance of lipid synthesis and accumulation in meat quality (Li et al., 2012), new interest has arisen towards the understanding of genetic mechanisms underlying such processes. With this goal, some studies based on the microarray technology investigated transcriptome differences between genotypes, as Iberian vs. Large White or Duroc pig in endocrine tissues (Pérez-Enciso et al., 2009). On the other hand, several studies have addressed the effect of the developmental stage (prenatally and postnatally) on muscle transcriptome (D'Andrea et al., 2011; Voillet et al., 2014; Zhao et al., 2015), or the effect of muscle type on transcriptome and proteome, showing important functional differences (Herault et al., 2014; Sobol et al., 2015). For example, 15 – 30% of proteome has been reported to differ between Longissimus dorsi (LD) and Biceps femoris (BF) muscles (Te Pas et al., 2011; Herault et al., 2014). Although LD is a muscle with high economic relevance, and up to now it has been examined in more detail and more frequently, the usefulness of the joint analysis of different muscles is evident, as proposed by Sobol et al. (2015).

Previous studies assessed transcriptomic differences between pure Iberian and Duroc-crossbred Iberian pigs using microarray technology in the loin (Ovilo et al., 2014b) and RNA-Seq technology in BF muscle (Ayuso et al., 2015b). However, this is the first RNA-Seq technology-based study focused on genetic differences between genotypes, developmental stages and muscle types aimed at improving the knowledge of the genetic and metabolic basis of meat quality and productive traits in Iberian pigs.

Hence, the present study analyzed the LD muscle of newborn and four months old IB and IBxDU pigs, and employed data previously obtained from BF muscle of the same newborn animals aiming to: 1) Address the effects of genetic type, muscle, age and their interactions on phenotypic parameters; 2) Evaluate changes in muscle gene expression conditional on age, genetic type and muscle, that might be responsible for the observed phenotypic differences and identify pathways and networks in which those genes are involved; 3) Identify transcription factors affecting gene expression in order to establish potential new candidate genes affecting productive parameters and meat quality in purebred and Duroc-crossbred Iberian pigs, that could become targets for future studies as genetic markers for selection.

3.2.3- Materials and methods

Ethics statement

Animal manipulations were done in compliance with the regulations of the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 about the protection of animals used in research. The experiment was specifically assessed and approved (report CEEA 2010/003) by the INIA Committee of Ethics in Animal Research, which is the named Institutional Animal Care and Use Committee (IACUC) for the INIA.

Animals and sample collection

A total of 16 pure Iberian (IB) and 20 Iberian x Duroc (IBxDU) male piglets were used, coming from Iberian sows mated with either Iberian or Duroc boars to obtain purebred and crossbred piglets. At birth, nine IB and 10 IBxDU piglets were sacrificed. The remaining pigs were subject tostandard productive management up to four months of age, when seven IB and 10 IBxDU were slaughtered. Blood samples were collected from newborns and four months old pigs in sterile heparin blood vacuum tubes (Vacutainer Systems Europe, Meylan, France). Immediately after recovery, the blood was centrifuged at 1500g for 15min and the plasma was separated and stored into polypropylene vials at −20°C until assayed for determination of glucose and lipids metabolism-indicating parameters. After blood collection, pigs were slaughtered. Several body measures were obtained with a measure-tape: total body length (from the rostral edge of the snout to the tail insertion), ham length (from the anterior edge of the *Symphysis pubica* to the *articulatio tarsi)*, total length of anterior and posterior limbs (from the distal edge of the hooves to the proximal edge of the *scapula* or *Symphysis pubica*, respectively); and thoracic, abdominal and ham circumferences. Carcasses were weighted and samples from LD muscle in newborn and four months old pigs were vacuum-packed in low-oxygen permeable film and kept frozen at –20°C until fatty acid composition analysis. Prior to fatty acid analysis, muscle samples were freeze dried for two days in a lyophilizer (Lyoquest, Telstar, Tarrasa, Spain) and grounded in a Mixer Mill MM400 (Retsch technology, Haan, Germany) until muscle was completely powdered. For transcriptomic analysis, LD samples were immediately frozen in liquid nitrogen and maintained at –80°C until RNA extraction

The metabolic status of the pigs was evaluated. Glucose, fructosamine, triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) plasmatic levels were measured with a clinical chemistry analyzer (Saturno 300 plus, Crony Instruments s. r. l., Rome, Italy).

Tissue composition analysis

Longissimus dorsi muscle IMF content was quantified using the method proposed by Segura and Lopez-Bote (2014) based on gravimetrical determination of lipid content. Fatty acid methyl esters (FAMEs) were identified by gas chromatography as described by López-Bote et al. (1997) using a Hewlett Packard HP-6890 (Avondale, PA, USA) gas chromatograph equipped with a flame ionization detector and a capillary column (HP-Innowax, 30 m × 0.32 mm i.d. and 0.25 μm polyethylene glycol-film thickness). Results were expressed as grams per 100 grams of detected FAMEs.

Statistical analyses of tissue composition

Phenotypic data were analyzed as a completely randomized design using the general linear model (GLM) procedure using SAS version 9.2 (SAS Inst. Inc., Cary, NC; 2009). The mean and genetic type/muscle/age were considered as systematic effects, and residual effects as random. Carcass weight was used as covariate when it was significant and removed from the model when it was not significant. The animal was the experimental unit for all analysis. The results were considered to be significant at p-value < 0.05.

Transcriptomic analysis

RNA extraction

A total of 24 animals were used to perform transcriptomic analysis (6 animals of each genetic type at each studied age). Total RNA was extracted from 50-100mg samples of LD muscle using the RiboPure TM of High Quality total RNA kit (Ambion, Austin, TX, USA) following the manufacturer's recommendations. RNA was quantified using a NanoDrop-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The quality of the RNA was evaluated using the RNA Integrity Number (RIN) value from the Agilent 2100 Bioanalyzer device (Agilent technologies, Santa Clara, CA, USA). The RIN values ranged from 7.8 to 9.8

Library construction and RNA sequencing

Sequencing libraries were made using the mRNA-Seq sample preparation kit (Illumina Inc., Cat. # RS-100-0801) according to manufacturer's protocol. Each library was sequenced using TruSeq SBS Kit v3-HS, in paired end mode with the read length 2x76bp on a on a HiSeq2000 sequence analyzer (Illumina, Inc). Images from the instrument were processed using the manufacturer's software to generate FASTQ sequence files.

Mapping and assembly

Sequence reads were analyzed using CLC Bio Genomic workbench software 7.0 (CLC Bio, Aarhus, Denmark). Quality control analysis was performed using the NGS quality control tool, which assesses sequence quality indicators based on the FastQC-project (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Quality was measured taking into account sequence-read lengths and base-coverage, nucleotide contributions and base ambiguities, quality scores as emitted by the base caller and over-represented sequences (Cánovas et al., 2014). All the samples analyzed passed all the QC parameters having the same length (76 bp), 100% coverage in all bases, 25% of A, T, G and C nucleotide contributions, 50% GC on base content and less than 0.1% over-represented sequences. A hierarquical clustering of the samples was also performed. Sequence paired-end reads (76bp) were assembled against the annotated Sscrofa10.2 reference genome (http://www.ncbi.nlm.nih.gov/genome/?term=sus+scrofa) using the genome, annotated genes and mRNA tracks. Data was normalized by calculating the 'reads per kilo base per million mapped reads' (RPKM) for each gene (Mortazavi et al., 2008).

Differential expression analysis

The statistical analysis was performed using the total exon reads as expression values by the Empirical analysis of differential gene expression tool. This tool is based on the EdgeR Bioconductor package (Robinson et al., 2010) and uses count data (i.e. total exon reads) for the statistical analysis. Genes were filtered according to two criteria: a minimum mean group expression greater than 0.5 RPKM in at least one group and a Fold-Change (FC) of the expression differences between genotypes, muscles and age equal or higher to 1.5. Finally, those genes with a *p*-value ≤ 0.01, corresponding to a false discovery rate (FDR) value ≤ 0.14 in newborns and ≤ 0.198 in four months old pigs, were considered as differentially expressed (DE). Gene expression data of BF muscle was previously analyzed (30) following the same criteria. Three different effects were considered for the differential expression study: age effect on LD muscle transcriptome, genetic type effect on LD muscle transcriptome (at both ages independently); and muscle type effect (LD vs BF).

To validate the global RNA-Seq results, the concordance correlation coefficient (CCC) (Miron et al., 2006) was calculated in the same pigs between the FC values estimated in BF muscle from RNA-Seq and qPCR expression measures for the 9 genes analyzed by the two technologies (RNA-Seq and qPCR) (Ayuso et al., 2015b).

Systems biology study

The biological interpretation of the DE genes observed in LD muscle was performed using two complementary approaches in order to identify: 1) enriched pathways and networks involving the DE genes, and 2) potential regulators causing the observed changes in gene expression.

Ingenuity Pathway Analysis, (IPA) (Ingenuity Systems, Qiagen, California) software was employed to identify and characterize biological functions, gene networks and canonical pathways affected by the DE genes.

Regulatory transcription factors (TRF), which could potentially affect the DE genes in the dataset were also studied by following complementary approaches. First, RIF1 and RIF2 metrics (Reverter et al., 2010; Hudson et al., 2012) were calculated for the whole set of DE genes obtained conditional on genetic type at birth (261 genes) and four months of age (113 genes). Candidate TRFs in pigs were obtained from Animal TFDB (http://www.bioguo.org/AnimalTFDB/BrowseAllTF.php?spe=Susscrofa). A total of 1,038 TRF were retrieved. Among them, 739 showed expression values greater than 0.5 RPKM in at least one experimental group at birth and 655 at four months of age and thus, were used in the RIFs metrics approach.

The RIF1 and RIF2 values were computed for the i^{th} TRF as follows:

$RIF1_i = \frac{1}{n_{de}} \sum_{j=1}^{j=n_{de}} \hat{a}_j \, x \hat{d}_j \left(r1_{ij} - r2_{ij}\right)^2$ and

$$RIF2_i = \frac{1}{n_{de}} \sum_{j=1}^{j=n_{de}} \left[\left(e1_j x r1_{ij}\right)^2 - \left(e2_j x r2_{ij}\right)^2\right]$$

where n_{de} is the number of DE genes, $\hat{a}_j$ and $\hat{d}_j$ the estimated average expression and differential expression of the j^{th} DE gene, $r1_{ij}$ and $r2_{ij}$ the co-expression correlation between the i^{th} TRF and the j^{th} DE gene in each one of the genetic types and being $e1_j$ and $e2_j$ the expression of the j^{th} gene in each genetic type (Almudevar et al., 2006). Both RIF measures for each analyzed TRF were transformed to standardized *z*-scores by subtracting the mean and dividing by its standard deviation. We identified relevant TRF as those with extreme RIF z-scores according to the corresponding confidence intervals (CI) calculated by bootstrap. In each iteration of bootstrapping, a set of n_{de}= 261 genes were randomly selected from the 12,245 expressed genes, and the RIF1 and RIF2 z-scores of the 739 TRF were calculated for newborn pigs. Similarly, in four months old pigs, a set of n_{de}= 113 genes were randomly selected from the 11,586 expressed genes, and the RIF1 and RIF2 z-scores of the 655 TRF were calculated. The procedure was repeated 10,000 times for each scenario to obtain the corresponding 95 and 99% CI intervals of both z-scores.

Complementarily, IPA software was employed to identify and characterize potential regulators using two different tools, the *upstream regulators* and the *regulators* tools. Both of them identify known regulators that may be affecting expression of the data set of DE genes. IPA-identified

regulators include genes, but also other molecules as drugs. Thus, in the regulators analysis concerning the genetic type effect, out of the identified regulators, only genes that were also included in the RIFs metrics candidate TRF list were considered (genes included in the animal TFDB and with expression values higher than 0.5 RPKM in at least one experimental group). However, in the two regulators analysis corresponding to the age and muscle effects, only regulators that were also DE were considered.

Using the information obtained from the TRF study, an additional search for enriched pathways and networks was carried out with IPA software considering both, DE genes and TRF regarding the genetic type effect.

3.2.4- Results

The transcriptome of muscle LD was studied at two different developmental stages, birth and four months of age. Moreover, the BF muscle transcriptome was previously assessed at birth in the same newborn pigs (Ayuso et al., 2015b). The experimental design allowed the study of several main effects regarding muscle transcriptome: age, genetic type and tissue effects were evaluated in the present study. The biggest transcriptome change was observed between the two studied ages, involving more than 5,800 DE genes (Table 1), followed by the genetic type effect, responsible for differential expression of 261 genes at birth and 113 at four months of age and the tissue effect, with 135 DE genes between LD and BF (Table 1).

Table 1: Differentially expressed (DE) genes as affected by the three studied main effects.

	Total DE genes	Upregulated genes	
		Birth	four months
Time effect	5812	3290	2522
		IBxDU[1]	IB[2]
Genotype effect at birth	261	131	130
Genotype effect at four months	113	25	88
		BF[3]	LD[4]
Tissue effect	135	52	83

[1] IBxDU = Iberian x Duroc crossbred pigs
[2] IB = Purebred Iberian pigs
[3] BF = Biceps femoris muscle
[4] LD = Longissimus dorsi muscle

Phenotypic results

Effect of age and terminal sire line on Iberian pig phenotype.

Age and genetic type significantly affected carcass characteristics, plasma biochemical parameters and meat quality traits, such as IMF and fatty acids profile (Table 2). Pure Iberian and crossbred piglets were slaughtered at birth at an average of 1.2 and 1.8 kg live weight, respectively (Table 2). Genetic type affected all the carcass phenotypic parameters at this early age as reported in a previous work employing the same newborn animals (Ayuso et al., 2015b): IBxDU neonates were bigger and heavier ($p < 0.001$) than IB newborns. On the other hand, four months old IB and IBxDU pigs slaughtered at 59.4 and 68.6 kg live weight, respectively, showed no difference in body weight or size, although ham weight and perimeter were higher in IBxDU than in IB pigs ($p = 0.039$ and $p = 0.034$, respectively). A significant interaction between age and genotype was observed for ham weight and circumference measures, due to the stronger genotype effect observed at birth.

Table 2: Effect of genotype, age and their interaction on phenotype of Iberian pigs.

	Genetic type (GT)								Age	GT*Age
	Birth				Four months of age					
Carcass traits	IBxDU[1]	IB[2]	SEM[3]	p-value	IBxDU[4]	IB[5]	SEM	p-value	p-value	p-value
Live weight	1.77	1.21	0.07	0.0008	68.60	59.40	2.41	0.0844	<.0001	0.0537
Carcass weight	1.41	0.96	0.05	0.0005	56.22	48.60	2.17	0.1095	<.0001	0.0742
Carcass lenght	40.20	35.50	0.54	0.0004	121.11	116.17	1.54	0.1402	<.0001	0.9359
Torax circumference	25.15	22.06	0.39	0.001	89.17	86.00	1.11	0.1858	<.0001	0.9737
Abdomen circumference	18.90	17.28	0.38	0.0486	77.28	78.17	1.21	0.7242	<.0001	0.2885
Ham weight	0.16	0.11	0.01	0.0007	7.56	6.23	0.29	0.039	<.0001	0,0171
Ham lenght	7.45	6.33	0.14	0.0009	25.89	24.33	0.36	0.0519	<.0001	0.5412
Ham circumference	12.55	10.89	0.23	0.002	67.83	59.42	1.74	0.0339	<.0001	0.0399
Lipid and glucose metabolism-related plasma indicators										
Cholesterol	72.12	102.36	7.64	0.0646	107.63	95.10	3.86	0.1356	0.1439	0.0305
LDL[6]	42.16	45.82	4.84	0.7103	67.38	61.45	2.58	0.2804	0.0019	0.4312
HDL[7]	22.38	41.20	4.83	0.0686	30.26	23.98	2.93	0.313	0.4519	0.0495
TG[8]	37.90	76.67	6.59	0.0092	46.41	53.71	4.03	0.439	0.3538	0.0258
Fructosamine	169.70	133.67	10.37	0.1009	221.33	228.00	8.05	0.6914	<.0001	0.1349
Glucose	132.40	123.44	10.80	0.6839	107.78	92.50	2.91	0.0233	0.036	0.8044
Longissimus dorsi muscle main fatty acids composition (g/100 g total fatty acids)										
IMF[9]	2.16	2.32	0.50	0.3168	2.87	4.05	0.20	0.0258	<.0001	0.6547
C14:0	2.77	2.56	0.12	0.4056	1.14	1.32	0.08	0.2948	<.0001	0.2304
C15:1	1.51	1.26	0.09	0.1706	0.60	0.33	0.04	0.026	<.0001	0.7431
C16:0	26.29	26.12	0.18	0.6558	24.56	25.44	0.39	0.1135	0.0013	0.0717

	Genetic type (GT)				Age				GT*Age	
	Birth				Four months of age					
	IBxDU[1]	IB[2]	SEM[3]	p-value	IBxDU[4]	IB[5]	SEM	p-value	p-value	p-value
C16:1 n-9	2.13	2.03	0.05	0.5472	0.20	0.27	0.02	0.0996	<.0001	0.2315
C16:1 n-7	5.93	5.40	0.22	0.2342	3.20	3.34	0.13	0.9382	<.0001	0.3566
C17:0	1.78	1.50	0.07	0.0644	0.47	0.32	0.03	0.0389	<.0001	0.1974
C17:1	0.94	0.88	0.05	0.5637	0.46	0.31	0.03	0.0418	<.0001	0.8618
C18:0	10.62	9.43	0.32	0.0834	12.91	12.69	0.23	0.7874	<.0001	0.1299
C18:1 n-9	24.06	26.61	0.80	0.1295	41.39	42.42	0.49	0.3733	<.0001	0.0854
C18:1 n-7	6.34	5.82	0.21	0.2352	2.67	2.21	0.13	0.0514	<.0001	0.6232
C18:2 n-6	7.00	8.76	0.56	0.1318	7.32	5.36	0.32	0.0203	0.0403	0.0215
C18:3 n-3	0.28	0.25	0.02	0.3506	0.18	0.18	0.01	0.8385	0.0012	0.3704
C18:4 n-3	0.13	0.17	0.02	0.3509	0.08	0.08	0.00	0.3474	0.0188	0.2219
C20:0	0.36	0.27	0.04	0.3199	0.20	0.19	0.01	0.9633	0.0307	0.5315
C20:1 n-9	0.63	0.60	0.05	0.7862	0.77	0.78	0.02	0.6352	0.0073	0.9574
C20:2 n-6	0.32	0.33	0.06	0.9132	0.61	0.45	0.04	0.0404	0.0235	0.1866
C20:3 n-6	0.63	0.52	0.02	0.0172	0.25	0.23	0.02	0.525	<.0001	0.0639
C20:4 n-6	5.65	4.97	0.27	0.2205	2.02	1.18	0.11	0.0071	<.0001	0.578
C22:4 n-6	1.07	0.91	0.05	0.1745	0.43	0.54	0.04	0.1924	<.0001	0.0481
C22:5 n-3	0.36	0.33	0.01	0.2535	0.34	0.85	0.04	<.0001	<.0001	<.0001
C22:6 n-3	0.31	0.26	0.04	0.5438	0.40	0.99	0.11	0.0197	0.0032	0.0117
∑SFA[10]	42.57	40.46	0.54	0.0678	39.32	40.13	0.55	0.1787	0.0087	0.0122
∑MUFA[11]	41.41	42.82	0.50	0.1744	49.50	49.01	0.64	0.4194	<.0001	0.1084
∑PUFA[12]	16.02	16.72	0.44	0.4407	11.73	9.71	0.50	0.1017	<.0001	0.2843
UI[13]	91.19	91.79	0.44	0.4407	79.37	81.39	1.58	0.7516	<.0001	0.9684
∑n-3[14]	1.35	1.22	0.91	0.7458	1.00	2.10	0.16	0.0036	0.1985	0.0012
∑n-6[15]	14.67	15.50	0.07	0.3243	10.73	7.65	0.47	0.0131	<.0001	0.0506
∑n-6/∑n-3	11.61	12.88	0.42	0.3443	14.31	3.89	1.22	0.0027	0.043	0.0003

[1] IBxDU = Iberian x Duroc crossbred pigs (n=10)
[2] IB = Purebred Iberian pigs (n=9)
[3] SEM = Standard error of the mean
[4] IBxDU = Iberian x Duroc crossbred pigs (n=10)
[5] IB = Purebred Iberian pigs (n=7)
[6] LDL = Low density lipoproteins
[7] HDL = High density lipoproteins
[8] TG = Triglycerides
[9] IMF = Intramuscular fat
[10] ΣSFA = Sum of saturated fatty acids
[11] ΣMUFA = Sum of monounsaturated fatty acids
[12] ΣPUFA = Sum of polyunsaturated fatty acids
[13] UI = Unsaturation index = 1 × (% monoenoics) +2 × (% dienoics) +3 × (% trienoics) +4 × (% tetraenoics) +5 × (% pentaenoics) +6 × (% hexaenoics)
[14] Σn3 = Sum of n-3 fatty acids
[15] Σn6 = Sum of n-6 fatty acids

Glucose levels decreased and fructosamine and LDL increased in four months old pigs of both genotypes, in comparison to newborns. Moreover purebred IB newborns had significantly greater

plasma levels of total and HDL cholesterol, and triglycerides (TG) than IBxDU neonates. An interaction was observed for these parameters, showing four months old IB and IBxDU similar cholesterol and TG levels. On the other hand, four months old pigs differed in plasma glucose: IBxDU showed higher levels than IB pigs at this age ($p = 0.023$).

Regarding IMF, the sums of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids were calculated. Total IMF content increased over time ($p < 0.0001$). Genetic type did not affect IMF content in LD muscle (Table 2) of newborn piglets, while IB showed a 41% increase in IMF content with respect to IBxDU pigs at four months of age ($p = 0.026$). Age also affected IMF composition: MUFA content increased and PUFA content decreased over time ($p < 0.0001$), while SFA content slightly decreased along growth in IBxDU but not in IB pigs due to an interaction between genotype and age ($p = 0.012$). Also, SFA content showed a trend ($p = 0.068$) for higher concentration in newborn IBxDU pigs, as observed for C20:3 concentration ($p = 0.017$). At four months of age, C18:2 C20:2 and C20:4 concentrations were significantly higher in IBxDU than in IB pigs ($p = 0.0203$, 0.0404 and 0.0071, respectively) while C22:5 and C22:6 proportions were higher in IB pigs ($p < 0.0001$ and $p = 0.020$, respectively). As a consequence, total n-3 fatty acids content was higher in IB ($p = 0.004$) and total n-6 and the ratio n-6/n-3 was higher in IBxDU pigs ($p = 0.013$ and $p = 0.003$, respectively).

Effect of muscle type, Biceps femoris or Longissimus dorsi, on intramuscular fat content and composition.

Data on BF phenotype and transcriptome was available for the same newborn animals from a previous study (Ayuso et al., 2015b). Thus, the effect of the studied muscle (BF *vs* LD) was assessed in IB and IBxDU neonates (Table 3). *Biceps femoris* and LD muscles showed similar IMF content at this early age ($p = 0.158$). Differences in total PUFA and n-3 PUFA content were observed, showing BF higher levels than LD muscle. Also, a decrease in the ratio n-6/n-3 was observed in BF muscle. When analyzing the global effect of genotype on IMF content and composition of both muscles, higher SFA and lower n-6 PUFA content was observed in IBxDU pigs. Moreover, trends were detected for higher ratio n-6/n-3 IMF and PUFA content in IB piglets ($p = 0.0564$, $p = 0.0597$, and $p = 0.0567$, respectively). No significant interaction was found between the genotype and muscle effects.

Table 3: *Longissimus dorsi* (LD) and *Biceps femoris* (BF) muscle characteristics of pure and Duroc-crossbred Iberian piglets at birth.

		Mean			p-value		
	Muscle	IBxDU[1]	IB[2]	SEM[3]	Genetic type (GT)	Muscle	GT*Muscle
∑SFA[4]	LD	42,57	40,79	0,33	0,0016	0,1821	0,5376
	BF	41,66	39,91				
∑MUFA[5]	LD	41,41	42,73	0,32	0,1142	0,4784	0,6531
	BF	41,01	41,77				
∑PUFA[6]	LD	16,02	16,48	0,31	0,0567	0,0394	0,8454
	BF	17,34	18,32				
∑n-3[7]	LD	1,35	1,24	0,04	0,1445	<.0001	0,9423
	BF	1,77	1,69				
∑n-6[8]	LD	14,67	15,24	0,30	0,0338	0,123	0,8333
	BF	15,56	16,63				
∑n-6/∑n-3	LD	11,61	12,54	0,36	0,0564	0,0023	0,8922
	BF	8,78	9,96				
IMF[9]	LD	2,16	2,26	0,08	0,0597	0,1582	0,6441
	BF	1,80	2,16				

[1] IBxDU = Iberian x Duroc crossbred pigs (n=10)
[2] IB = Purebred Iberian pigs (n=9)
[3] SEM = Standard error of the mean
[4] ΣSFA = Sum of saturated fatty acids
[5] ΣMUFA = Sum of monounsaturated fatty acids
[6] ΣPUFA = Sum of polyunsaturated fatty acids
[7] Σn3 = Sum of n-3 fatty acids
[8] Σn6 = Sum of n-6 fatty acids
[9] IMF = Intramuscular fat

Transcriptome analysis

Mapping results

An average of approximately 84 million sequence reads was obtained for each individual sample and were assembled and mapped to the annotated Sscrofa10.2 genome assembly (22,861 genes). In all samples, 67-77% of the reads were categorized as mapped reads to the porcine reference sequence. The RPKM values were used to establish the total number of genes expressed in muscle transcriptome (>0.5 RPKM). Approximately 50 % of total porcine annotated genes in the Sscrofa10.2 genome assembly were expressed in the studied samples (an average of 11,506 genes out of 22,861 annotated genes).

Effect of age on Longissimus dorsi transcriptome

A total of 3,290 genes were upregulated in LD muscle at birth when compared to four months-old pigs ($p < 0.01$, FDR < 0.015), with FC ranging from 1.5 to 219 (*IBSP* gene showed the largest expression difference). In four months-old pigs, 2,522 genes were upregulated when compared to

newborn pigs. Fold changes (FC) ranged from 1.5 to 273, with several immunoglobulin genes showing the biggest expression changes (Table S1).

Gene expression differences were functionally interpreted using IPA software to detect enriched pathways (Table S2) and biological functions (Table 4). Moreover, 99 DE genes were identified as potential regulators affecting expression of other DE genes in the dataset (i.e. *MSTN, FOXO3, FOXO1, MEF2C* or *MEF2D*) (Table S3).

Table 4: Enriched biological functions in the set of DE genes between LD muscle from newborn and four months old Iberian pigs

Upregulated at birth			Upregulated at four months		
Enriched function	P-value	Z-score[1]	Enriched function	p-value	z-score
Invasion of cells	6,80e-13	3,93	Organismal death	2,35E-26	6,95
Size of body	1,33e-08	3,58	Growth failure	1,10E-06	4,94
Transport of molecule	6,25e-11	3,33	Bleeding	2,14E-05	4,93
Invasion of tumor cell lines	9,56e-09	3,18	Perinatal death	4,07E-08	3,92
Cell movement	1,63E-20	3,09	Contractility of skeletal muscle	7,09E-06	3,86
Cell movement of tumor cell lines	1,40E-09	3,05	Hypoplasia of organ	1,79E-06	3,26
Apoptosis of fibroblast cell lines	2,32e-10	3,03	Hypoplasia	1,26E-06	3,15
Adhesion of connective tissue cells	2,34e-05	2,94	Dysgenesis	6,61E-07	3,04
Cancer	7,49e-58	2,94	Congenital anomaly of musculoskeletal system	4,56E-10	2,92
Migration of cells	1,47e-17	2,90	Multiple congenital anomalies	1,55E-07	2,91
Migration of tumor cell lines	2,79e-09	2,77	Polymerization of protein	1,07E-06	2,73
Limb development	2,75E-06	2,77	Fibrosis	2,37E-05	2,39
Invasion of tumor	2,62e-09	2,70	Hypoplasia of thorax	2,54E-05	2,25
Proliferation of fibroblasts	3,70e-07	2,59	Autophagy	1,33E-07	2,18
Adhesion of tumor cell lines	8,98e-07	2,58	Anemia	9,38E-10	1,84
Disassembly of filaments	4,34e-05	2,56	Mass of muscle	2,10E-05	1,77
Synthesis of DNA	2,84E-06	2,56	Adhesion of extracelular matrix	6,49E-07	1,73
Invasion of breast cáncer cell lines	2,08e-05	2,56	Autosomal recessive disease	9,73E-11	1,72
Synthesis of carbohydrate	3,57e-05	2,52	Mass of skeletal muscle	5,47E-05	1,69
Tumorigenesis of tissue	3,36e-51	2,46	Blood vessel defect	5,38E-06	1,56
Neoplasia of cells	3,92e-06	2,45	Systemic autoinmune syndrome	4,06E-05	1,53
Invasion of tumor cells	8,87e-08	2,40	Dwarfism	3,13E-07	1,46
Organization of cytoplasm	3,88e-19	2,36	Abnormal bone density	3,32E-05	1,45
Metabolism of carbohydrate	3,20e-08	2,36	Dysplasia of skeleton	1,13E-06	1,38
Endocytosis	3,23e-07	2,33	Aneurysm	1,83E-05	1,38
Proliferation of neuronal cells	3,09e-09	2,30	Vascular tumor	8,82E-07	1,33
Organization of cytoskeleton	2,72e-18	2,28	Hemangioma	1,06E-05	1,33
Development of central nervoussystem	2,12e-06	2,26	Hypertension	2,50E-11	1,33
Development of body trunk	5,08e-11	2,22	Hypoglycemia	6,77E-06	1,24
Outgrowth of cells	8,82e-06	2,19	Adhesion of cell-associated matrix	3,14E-05	1,19
Neoplasia of epitelial tissue	1,26e-51	2,18	Ulcer	1,04E-05	1,13
Formation of celular protrusions	9,48e-11	2,18	Chronic inflammatory disorder	2,84E-07	1,06
Microtubule dynamics	1,79E-16	2,16	Replication of virus	5,01E-06	1,01
Upregulated at birth			Upregulated at four months		
Behavior	5,08e-06	2,12	Quantity of muscle cells	1,26E-09	0,99
Cell death of fibroblasts	4,70E-06	2,11	Mass of hind limb muscle	8,61E-06	0,91
Proliferation of cells	1,95e-40	2,10	Function of muscle	1,04E-07	0,89
Invasion of malignant tumor	1,07e-08	2,10	Breast or ovarían cancer	3,20E-09	0,86
Development of cardiovascular system	1,19e-20	2,06	Quantity of muscle	3,44E-09	0,86

[1]Z-score: Predicted activation status of biological function. The higher the value, the more activated the functions is predicted to be.

Effect of genetic type on Longissimus dorsi transcriptome

The effect of genetic type on LD muscle transcriptome was analyzed at birth and at four months of age independently, due to the high influence of age on gene expression. Genetic type effect on gene expression seemed to be stronger at birth, since 261 genes were DE ($p < 0.01$, FDR < 0.141) at that young age while less than a half, 113 DE genes were identified at four months of age ($p < 0.01$, FDR < 0.199) between IN and IBxDU pigs. Out of the 261 DE genes observed at birth, 130 were upregulated in IB (FC from 1.8 to 25.6) and 131 in IBxDU pigs (FC from 1.8 to 58.5). At four months of age, 83 genes were upregulated in IB (FC from 2.1 to 390) and 25 in IBxDU pigs (FC from 2.2 to 88.4) (Table S4).

Biological interpretation of the DE genes with IPA software retrieved enriched pathways and biological functions in IB and IBxDU pigs at both studied ages (Tables 5 and 6, respectively). Moreover, the regulators analysis, performed by combining information from IPA software and RIFs metrics, identified 122 TRF at birth and 62 TRF at four months of age, potentially regulating the gene expression changes observed between genetic types (Table S5). Sixteen of those TRF (such as *ATF4, CEBPA, MYOD1, NFE2L2* or *REL*) were found at both ages. Twelve regulators (*CREB3L1, CREBBP, FOXO1, HSF1, KLF1, MEF2C, MEF2D, MYOG, NFE2, SF1, SOX4* and *TEAD3*) were identified using the two approaches at birth and 3 (*EN1, IRF2* and *TCF7L2*) at four months of age. Moreover, a combined analysis performed using IPA software by merging DE genes and TRF datasets revealed the *glucocorticoid receptor signaling* and *adipogenesis* as the most enriched pathways at birth, while the *aryl hydrocarbon receptor* pathway was the most enriched in four months old pigs. The *PPAR signaling* and the *unfolded protein response* pathways were enriched at both stages (Table S6).

Table 5: Enriched pathways in the set of DE genes conditional on genetic type at birth and four months of age

Birth		4 months of age	
IB[1]	p-value	IB	
Role of IL-17A in Psoriasis	2,83E-05	Serine Biosynthesis	5,57E-09
PI3K Signaling in B Lymphocytes	6,56E-05	Superpathway of Serine and Glycine Biosynthesis I	1,29E-08
ERK5 Signaling	7,65E-05	Glutathione-mediated Detoxification	1,05E-04
Melatonin Degradation III	1,87E-04	LPS/IL-1 Mediated Inhibition of RXR Function	1,09E-04
Glutamine Biosynthesis I	1,87E-04	Alanine Degradation III	3,01E-04
NRF2-mediated Oxidative Stress Response	6,00E-04	Alanine Biosynthesis II	3,01E-04
IL-8 Signaling	6,92E-04	Cell Cycle: G1/S Checkpoint Regulation	2,02E-03
April Mediated Signaling	1,47E-03	Cyclins and Cell Cycle Regulation	4,96E-03
Trehalose Degradation II (Trehalase)	1,81E-03	Glucose and Glucose-1-phosphate Degradation	6,29E-03
B Cell Activating Factor Signaling	1,81E-03	p53 Signaling	1,62E-02
LXR/RXR Activation	2,80E-03	Leukotriene Biosynthesis	3,71E-02
Atherosclerosis Signaling	3,13E-03	LXR/RXR Activation	4,97E-02
TNFR1 Signaling	4,68E-03		
Wnt/Ca+ pathway	9,09E-03		
Glucocorticoid Receptor Signaling	1,17E-02		
GDP-glucose Biosynthesis	1,52E-02		
Glucose and Glucose-1-phosphate Degradation	2,27E-02		
Growth Hormone Signaling	2,61E-02		
Melatonin Signaling	2,80E-02		
UDP-N-acetyl-D-galactosamine Biosynthesis II	3,22E-02		
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	3,99E-02		
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	4,97E-02		
Birth		4 months of age	
IBxDU[2]		IBxDU	
Aldosterone Signaling in Epithelial Cells	1,11E-10	Acute Phase Response Signaling	3,90E-05
Protein Ubiquitination Pathway	1,71E-09	Retinoate Biosynthesis II	9,68E-05
Unfoldedprotein response	5,94E-08	Bupropion Degradation	1,03E-03
eNOSSignaling	6,94E-06	Acetone Degradation I (to Methylglyoxal)	1,47E-03
Endoplasmic Reticulum Stress Pathway	1,79E-04	Retinoate Biosynthesis I	2,02E-03
Pyrimidine Ribonucleotides Interconversion	4,74E-04	Retinol Biosynthesis	2,80E-03
Glucocorticoid Receptor Signaling	5,45E-04	Estrogen Biosynthesis	3,51E-03
Pyrimidine Ribonucleotides De Novo Biosynthesis	6,29E-04	Nicotine Degradation III	5,26E-03
Neuregulin Signaling	6,29E-04	Melatonin Degradation I	8,03E-03

Birth		4 months of age	
IBxDU[2]		IBxDU	
Role of p14/p19ARF in Tumor Suppression	7,27E-04	Nicotine Degradation II	9,68E-03
SpermineBiosynthesis	8,41E-04	IL-17 Signaling	1,10E-02
Alanine Degradation III	8,41E-04	GABA Receptor Signaling	1,10E-02
Alanine Biosynthesis II	8,41E-04	Superpathway of Melatonin Degradation	1,33E-02
Hepatic Fibrosis / Hepatic Stellate Cell Activation	2,02E-03	IL-6 Signaling	4,29E-02
Spermidine Biosynthesis I	2,02E-03		
PI3K/AKT Signaling	4,96E-03		
Serine Biosynthesis	7,10E-03		
Thioredoxin Pathway	1,17E-02		
Aryl Hydrocarbon Receptor Signaling	1,17E-02		
Epithelial Adherens Junction Signaling	1,62E-02		
Superpathway of Serine and Glycine Biosynthesis I	1,85E-02		
PCP pathway	2,27E-02		
Hypoxia Signaling in the Cardiovascular System	2,80E-02		
Mitotic Roles of Polo-Like Kinase	3,00E-02		

x
[1]

IB = Purebred Iberian pigs
[2]IBxDU = Iberian x Duroc crossbred pigs

Table 6: Enriched biological functions in the set of DE genes conditional on genetic type, at birth and four months of age

Birth			4 months of age		
IB[1]					
Function	P-value	Z-score[2]	Function	p-Value	z-score
Cell death of tumor cell lines	2.49E-06	-2.82	Size of body	7.37E-03	-2.35
Apoptosis	2.05E-09	-2.60	Quantity of connective tissue	3.22E-03	-1.98
Apoptosis of tumor cell lines	1.16E-07	-2.57	Lesion formation	2.50E-04	-1.35
Degradation of protein	2.63E-03	-2.57	Concentration of lipid	2.45E-04	-1.13
Necrosis	3.15E-07	-2.54	Concentration of fatty acid	6.70E-03	-1.09
Cell death	1.46E-10	-2.49	Activation of macrophages	1.26E-02	-1.09
Inflammatory response	2.21E-03	-2.47	Size of lesion	2.94E-03	-0.74
Accumulation of myeloid cells	7.89E-03	-2.42	Size of bone	1.20E-02	-0.68
Colony formation of tumor cell lines	1.49E-03	-2.39	Proliferation of cells	1.24E-02	-0.57
Proliferation of tumor cells	7.12E-03	-2.35	Concentration of triacylglycerol	1.45E-03	-0.41
Synthesis of reactive oxygen species	2.91E-04	-2.35	Quantity of reactive oxygen species	8.63E-03	-0.28
Colony formation of cells	3.24E-04	-2.30			
Accumulation of neutrophils	5.54E-04	-2.20			
Killing of bacteria	3.49E-04	-2.19			
Generation of reactive oxygen species	9.50E-04	-2.13			
Atrophy of muscle	2.97E-03	-2.11			
Differentiation of connectivetissue	1.24E-03	-2.05			
Metastasis of melanoma celllines	1.47E-04	-2.00			
IBXDU[3]					
Quantity of erythroid progenitor cells	2.14E-03	2.19	Organismal death	5.13E-03	2.61
Cytolysis	2.99E-05	2.18	Necrosis of epitelial tissue	3.53E-03	1.45
Engulfment of cells	3.67E-03	2.05	Metabolism of reactive oxygen species	1.09E-02	1.26
Morphology of cells	7.37E-05	2.05	Neuronal cell death	8.40E-03	1.20
Mean corpuscular hemoglobin concentration	4.14E-04	2.00	Transport of molecule	1.21E-02	1.15
Cellviability of tumor celllines	4.72E-03	1.99	Oxidation of lipid	2.74E-04	1.12
Extension of neurites	3.92E-03	1.98	Celldeath of epithelialcells	4.45E-03	1.11
Cell viability	8.69E-05	1.97	Colony formation of tumor celllines	1.39E-02	1.10
Quantity of reticulocytes	1.99E-03	1.96	Hydrolysis of triacylglycerol	7.45E-06	1.09
Cytosis	5.19E-03	1.94	Morbidity or mortality	1.15E-02	1.06
Cell survival	3.18E-05	1.86	Inflammation of body cavity	6.07E-03	1.00
Anemia	1.58E-05	1.80	Cell death	5.47E-04	0.98
Phagocytosis of cells	1.49E-03	1.80	Cancer	7.95E-03	0.82
Hyperplasia of epidermis	8.90E-05	1.71	Hypertrophy	1.08E-02	0.80

Birth			4 months of age		
	IBxDU[3]				
Size of cells	8.43E-03	1.60	Cell death of central nervous system cells	7.74E-03	0.79
Binding of cells	2.60E-03	1.49	Mineralization of cells	1.06E-04	0.76
Binding of granulocytes	2.43E-04	1.47	Apoptosis	3.24E-03	0.73
Mass of muscle	1.96E-03	1.38	Inflammation of organ	2.73E-03	0.71

[1]IB = Purebred Iberian pigs
[2]Z-score: Predicted activation status of biological function. The higher the value, the more activated the functions is predicted to be.
[3]IBxDU = Iberian x Duroc crossbred pigs

Effect of muscle type, Biceps femoris or Longissimus dorsi, on gene expression

Differences in gene expression were observed between LD and BF muscles, 83 genes showing higher expression levels in LD than in BF muscle (FC from 1.7 to 27.0), while 52 genes were upregulated in BF muscle (FC from 1.8 to 183.2) ($p < 0.01$, FDR < 0.123).Genes such as HOXA11, PVALB or CXCL13 (upregulated in BF muscle) and IBSP, ZIC1 and MMP13 (upregulated in LD muscle) showed the largest expression differences (Table S7).

IPA software was used to detect enriched pathways (Table S8) and biological functions (Table 7) associated with the DE genes between both muscles. Moreover, 3 DE genes were identified as potential regulators affecting expression of other DE genes in the dataset (SIM1, HOXC8 and HOXA10).

Table 7: Enriched biological functions in the set of DE genes conditional on muscle: *Longissimus dorsi* (LD)vs. *Biceps femoris* (BF), at birth.

Upregulated in LD			Upregulated in BF		
Enriched function	p-value	z-score[1]	Enriched function	p-value	z-score
Inflammation of organ	1,03E-03	2,678	Efflux of cholesterol	2,77E-04	-2,224
Proliferation of cells	9,33E-04	2,566	Muscle contraction	1,10E-04	-2,190
Activation of mononuclear leukocytes	6,74E-05	2,554	Binding of cells	2,01E-10	-2,071
Cell movement of monocytes	3,97E-05	2,408	Damage of genitourinary system	1,27E-03	-2,000
Size of body	6,07E-04	2,378	Calcinosis	7,89E-07	-1,982
Activation of lymphocytes	7,78E-04	2,378	Growth of organism	1,17E-03	-1,977
Activation of cells	5,49E-07	2,206	Quantity of connective tissue	3,87E-05	-1,792
Accumulation of leukocytes	6,28E-07	1,982	Hypoplasia of organ	5,80E-04	-1,773
Killing of bacteria	6,47E-08	1,968	Dysgenesis	7,80E-04	-1,773
Migration of leukemia cell lines	7,34E-04	1,934	Organismal death	2,22E-05	-1,390
Killing of E. Coli	2,84E-06	1,934	Binding of leukocytes	6,79E-09	-1,368
Neovascularization	2,14E-03	1,932	Binding of granulocytes	2,75E-08	-1,236
Accumulation of cells	1,87E-06	1,922	Binding of gonadal celllines	9,40E-05	-1,214
Activation of leukocytes	3,56E-06	1,830	Binding of bloodcells	5,79E-09	-1,206
Development of cardiovascular system	1,02E-07	1,820	Size of bone	1,47E-03	-1,204
Migration of cells	3,04E-07	1,817	Synthesis of nitric oxide	1,44E-03	-1,161
Migration of tumor cell lines	1,79E-03	1,815	Phagocytosis of cells	3,67E-05	-1,055
Activation of blood cells	9,99E-08	1,760	Quantity of carbohydrate	4,83E-04	-1,040
Activation of connective tissue cells	1,21E-04	1,746			
Quantity of neutrophils	6,33E-05	1,746			
Vascularization	4,74E-04	1,728			
Cell movement of mononuclear leukocytes	7,68E-04	1,534			
Accumulation of phagocytes	1,00E-07	1,521			
Viral infection	1,57E-03	1,505			
Vasculogenesis	2,75E-05	1,500			

[1]Z-score: Predicted activation status of biological function. The higher the value, the more activated the functions is predicted to be.

3.2.5- Discussion

Phenotypic results

Effect of age and terminal sire line on Iberian pig phenotype.

Age affected carcass characteristics, as expected: all measured parameters increased along time (Table 2). On the other hand, genetic type affected all the carcass phenotypic parameters in newborns: IBxDU were bigger and heavier ($p < 0.001$) than IB piglets (Table 2). However, at four months old, IB and IBxDU pigs only differed in ham weight and circumference, but no significant difference was found in other body size measures. Differences in ham measures between genotypes

observed in juvenile pigs agree with results obtained in pure and Duroc-crossbred Iberian pigs at final slaughter weight (Serrano et al., 2008; Robina et al., 2013). Previous works also reported that adult Duroc-crossbred Iberian pigs are longer and heavier than their purebred Iberian counterparts (Serrano et al., 2008; Robina et al., 2013), but these differences in body weight and size between genotypes are not evident at weaning (Ovilo et al., 2014b). Iberian pigs have a lower prenatal growth potential than lean pigs (Torres-Rovira et al., 2013). Thus, the finding of IB newborns being smaller in body size and weight than IBxDU is expected, due to the effect of the leaner and more growth-efficient Duroc sire. Postnatally, the thrifty genotype, characteristic of the Iberian breed might led to differences in voluntary feed intake and energy expenditure between IBxDU and IB pigs (Ovilo et al., 2014c) that could lead to a catch up growth in the latter during the suckling period. This compensatory growth would not be reflected on adult pigs due to a much lower growth potential of pure Iberian animals in later stages. This is an interesting hypothesis that would require further studies specially designed to analyze the evolution of growth and energy balance in different genotypes along early and juvenile growth.

Regarding biochemical parameters, plasma glucose levels were higher in neonates, probably because four months old animals were slaughtered after a fasting period, which may cause a depletion of plasma glucose levels. At four months of age, IBxDU pigs showed higher plasma glucose levels than IB pigs, in agreement with the reported decreased plasma glucose levels in obese when compared to lean fasted pigs, probably because of a high rate of glucose utilization for fat synthesis in obese pigs (He et al., 2012). Fructosamine levels were higher in older pigs, reflecting higher glucose levels during the previous 1-3 weeks (Johnson et al., 1983). In agreement, fructosamine has been previously reported to increase with age in Iberian sows (Torres-Rovira et al., 2011).

Certain lipid metabolism-related parameters (cholesterol, HDL and triglycerides) were significantly different in IB and IBxDU newborns, but seemed to balance in four months old pigs, with a significant interaction observed between age and genotype for these parameters: newborn IB pigs showed greater plasma cholesterol, HDL and triglycerides than CR piglets (Ayuso et al., 2015b), but these differences disappeared over time. The evolution over life span of lipid related plasma indicators has not been previously compared between different pig breeds. However, in a study assessing plasma biochemical parameters in wild boars, piglets (< 6 months of age) showed slightly higher cholesterol and doubled triglyceride levels than wild boars over 6 months of age (Casas-Díaz et al., 2015), while an increase as pigs mature in the concentrations of cholesterol has been reported in lean pigs (Klem et al., 2010). Thus, a different regulation of lipid metabolism in swine breeds over life might be responsible for the observed differences in cholesterol, HDL and triglycerides.

Intramuscular fat content was also affected by pigs' growth. Total IMF content increased over time ($p < 0.0001$) in both genetic types (table 2). In newborns, no differences on IMF content were observed between IBxDU and IB pigs, in contrast with results reported for muscle BF of newborn IB piglets, which showed almost 30% more IMF than BF of IBxDU pigs (Ayuso et al., 2015b). This supports that regulation of IMF deposition depends, among other factors, on muscle type (Muriel et al., 2004; Ayuso et al., 2015a). However, four months old IB pigs showed significantly greater IMF content than four months old IBxDU pigs in LD muscle, in concordance with the characteristic difference between genetic types in adult animals (Ventanas et al., 2006) (Table 2).

Regarding IMF composition, MUFA content increased over time ($p < 0.0001$) in both genetic types. The raise in MUFA content was due to an increase in C18:1 n-9, the major fatty acid. Thus, age-related changes led to a more fatty and monounsaturated meat in four months old when compared to newborn IB and IBxDU pigs. Moreover, the observed change in the fatty acids composition is concordant with the values previously observed in four months old Iberian pigs (Ayuso et al., 2015d) and with the ongoing acquisition of the characteristic fatty acids profile of Iberian products. Regarding the genetic type effect on IMF composition, no difference was observed between genetic types at birth, while a slight effect was observed at four months of age. The most remarkable effect was an increase in n-3 fatty acids and a decrease in the ratio n-6/n-3 in IB when compared to IBxDU. It has been previously reported that a decrease in the ratio n-6/n-3 and increase of C22:5 (DPA) and C22:6 (DHA) fatty acids promote consumers' health (Simopoulos, 2008; Mozaffarian and Wu, 2011). Moreover, the World Health Organization recommended the consumption of meat with the ratio n-6/n-3 below 5 (Who and Consultation, 2003). These results indicate that crossing with Duroc sires decreased meat quality in terms of consumers' health and IMF concentration in LD muscle of four months old pigs, in agreement with differences observed in weaned and adult pigs (Ventanas et al., 2006; Ovilo et al., 2014b).

Effect of muscle type on intramuscular fat content and composition.

We observed higher PUFA content in BF muscle, which was in part due to increased n-3 PUFA content in this muscle. This also led to a decrease in the ratio n-6/n-3. It is known that the pattern of fatty acids deposition may differ across muscles (Sharma et al., 1987; Leseigneur-Meynier and Gandemer, 1991; Kim et al., 2008). In agreement with the results of the present study, the biggest difference previously observed between LD and BF muscle of lean pigs was reported for PUFA content (Sobol et al., 2015), which might be due to the differences in oxidative properties observed between muscles (Karlsson et al., 1993; Andrés et al., 2001). As previously discussed, a lower ratio n-6/n-3 has been associated with healthier meat. Moreover, when analyzing jointly the data of both muscles, the inclusion of Duroc on Iberian genetics significantly increased SFA content in muscle of IBxDU newborns (Table 7), in accordance with previous results (Ventanas et al., 2006). High SFA

consumption increases cardiovascular disease risk in human (Simopoulos, 2004). Thus, meat from BF muscle of pure Iberian, may be considered healthier than meat from LD muscle of Duroc-crossbred Iberian pigs.

Transcriptome analysis

Effect of age on Longissimus dorsi transcriptome

The age of sampling was the main factor affecting gene expression in pure and Duroc-crossbred Iberian pigs, as 5,800 genes changed their expression levels between both developmental stages. Gene expression has been previously reported to change across age in pigs, especially during early stages of prenatal and postnatal development (Zhao et al., 2011). Some of the genes showing the highest upregulation in newborn piglets were involved in the development of different tissues, such as bone, cartilage, adipose or muscle tissues (*ACTC1, ARHGAP36, IBSP, TNN, ATP6V0D2, COMP*, *FGF21, DLK1* and several myosin and collagen proteins). Moreover, two genes associated with meat quality were highly upregulated at birth: RETN is associated with adipogenesis and lipogenesis and TNN is involved in the development of the extracellular matrix in pigs (Kayan et al., 2011; Čepica et al., 2012). Genes highly upregulated in four months old pigs were associated with the immune response (since several immunoglobulin genes such as *IGLC, IGLV7, 8, 9* and *10*, were identified), but also with protein metabolism (*PVALB, UBD*). Moreover, carrier proteins such as *TTR, HRG, ALB* or *APO*s were among the most upregulated genes in LD muscle from four months old pigs. The DE analysis suggests that different processes are active in each developmental stage. To verify this idea, a functional interpretation of the DE genes upregulated in each age was carried out using IPA software. Several metabolic pathways were enriched at both developmental stages, most of them involved in muscle growth (*Wnt/β-catenin Signaling, Calcium Signaling, Signaling by Rho Family GTPases or Actin Cytoskeleton Signaling*), in agreement with the studied tissue and growth period. Pathways enriched in newborn piglets are involved in cholesterol, triglycerides and other compounds (D-myo-inositol, chondroitin, phosphatidyl glycerol) biosynthesis, characteristic of a highly proliferative developmental stage (DeBerardinis et al., 2007; Vander Heiden et al., 2009). This is concordant with other enriched functions related to cellular growth and anabolic processes (as *invasion of cells, transport of molecule, cell movement, adhesion of connective tissue cells, proliferation of fibro blasts* and *synthesis of carbohydrate*). Contrary, normal non-proliferating or differentiated cells primarily utilize nutrients to fuel basic cellular processes and predominantly mediate catabolic metabolism to efficiently generate ATP (DeBerardinis et al., 2007; Vander Heiden et al., 2009), as observed in four months old pigs. At this age, enriched pathways were mainly related to catabolic processes (*Protein Ubiquitination Pathway, Glycolysis I, Gluconeogenesis I, Glucocorticoid Receptor Signaling* and *Phospholipase C Signaling*). Biological functions enriched at four months of age represent a decrease in developmental and growth processes that take place at

this age when compared to newborn piglets. Some of these enriched functions are: organismal death, growth failure, perinatal death, hypoplasia and dysgenesis. Moreover, functions related to muscle deposition and functioning (contractility of skeletal muscle, mass of muscle, quantity of muscle cells and function of muscle) appeared enriched at four months of age, in agreement with a more advanced muscle development than in newborn piglets (Zhao et al., 2011).

Changes in gene expression and thus, phenotypic consequences, between developmental stages were predicted to be regulated by a number of TRF identified using IPA software. Some of them were of special interest due to their influence in muscle and adipose growth. Identified regulators affecting muscle development include the *MSTN* gene, a known inhibitor of muscle growth (McPherron et al., 1997), and the myogenesis regulators *MYOD1*, *MEF2C* and *MEF2D*, essential ones at different stages of muscle growth (Edmondson et al., 1992; Ivana et al., 2005; Zhao et al., 2011). The forkhead family members *FOXO1* and *FOXO3* play a role in muscle but also in adipose tissue development (Allen and Unterman, 2007; Hakuno et al., 2011; Gupta et al., 2013; Jaitovich et al., 2015), while *PPARG*, *PPARGC1B*, *SIM1*, *ATF4* and *CEBPD* regulate adipocyte differentiation, energy homeostasis and lipid metabolism (Cao et al., 1991; Rosen et al., 1999; Franks et al., 2014; Tolson et al., 2014; Yu et al., 2014). These TRF potentially regulate muscle and fat accretion in young pigs and are thus, of special interest in the understanding of molecular mechanisms underlying such processes in pigs and other species.

Effect of genetic type on Longissimus dorsi transcriptome

Genetic type significantly affected gene expression at both developmental stages. Nine known genes (*GPT2, PSAT1, ART5, ADAMTS8, KCNH2, RASSF9, TP63, ENV* and *ASB5*) were found DE between genotypes at both developmental stages, suggesting an important role of these genes in the development of the IB and IBxDU phenotypes. Interestingly, three of them (*GPT2*, *PSAT1* and *ADAMTS8*) were differentially regulated at both ages, being upregulated in IBxDU at birth and in IB at four months. *GPT2* is involved in gluconeogenesis, fatty acids oxidation and amino acid metabolism in muscle and other tissues (Aagaard-Tillery et al., 2008; Marion et al., 2013), while *PSAT1* catalyzes serine biosynthesis. *ADAMTS8* has an important role in inhibition of angiogenesis (Dunn et al., 2006). Expression changes observed in these genes, related to muscle growth and metabolism may suggest a differential muscle growth regulation depending on age and genetic type.

Differentially expressed genes and enriched pathways and functions were of special interest when related to processes that may drive phenotypic differences observed between IB and IBxDU pigs. We found two main processes that seemed to be affected by the genetic type:

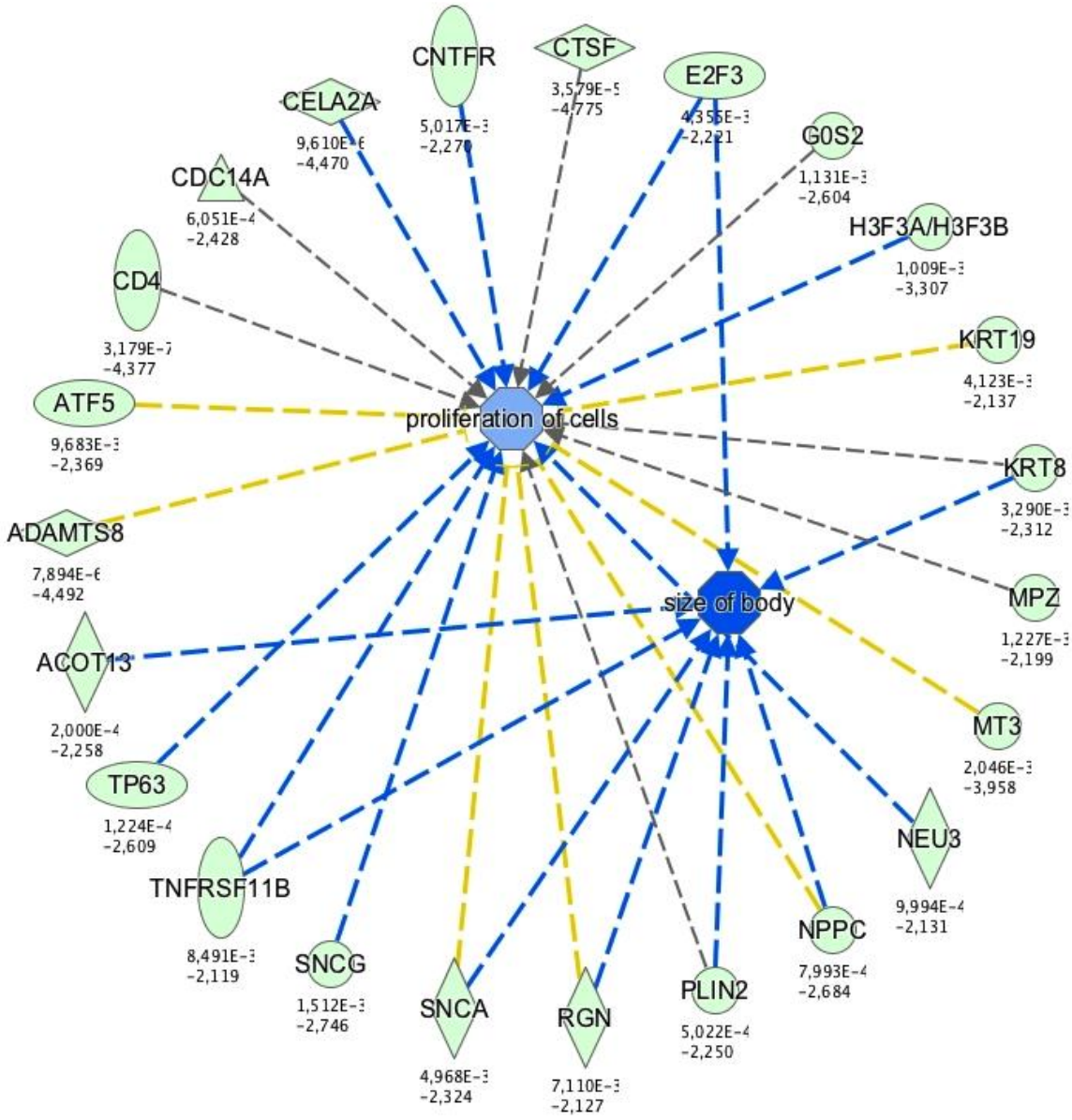
<u>Muscle growth</u>

Genes showing strong upregulation in newborn IBxDU pigs were associated with the extracellular matrix structure (*MATN1* and 3 or *COL9A1* and 2), connective tissue and muscle development (*GDF5, MYH10*) and also with protein metabolism and degradation (*PVALB, HSPs*).*MYH10* was also upregulated in IBxDU pigs in two previous studies comparing muscle transcriptome of newborn (Ayuso et al., 2015b) and weaning (Ovilo et al., 2014b) IB and IBxDU pigs, indicating a relevant role for this gene in muscle development differences between genotypes. These results are concordant with the greater prenatal development and with the enriched functions (Table 5) in crossbred newborns. For example, the *PI3K/AKT Signaling* pathway, involved in muscle growth (Briata et al., 2012), was enriched in IBxDU when compared to IB newborns.

In four months old pigs, pathways involved in the metabolism of non-essential amino acids such as serine, glycine and alanine were enriched in the IB genotype. Those amino acids are necessary for synthesis of proteins and other biomolecules needed for cell proliferation, including nucleotides, phosphatidyl-serine and sphingosine (Possemato et al., 2011), suggesting an active protein synthesis in four months old IB pigs. In accordance with this, several genes involved in *body size* and *cell proliferation* were upregulated in IB pigs (Fig 1), probably associated with the increased body growth that the smaller IB neonates may suffer and led to observed similar body weight in four months old IB and IBxDU pigs, as previously hypothesized.

Fig 1:Enriched biological functions related to body growth in four months old IB pigs.

The network generated by IPA software shows enriched biological functions in IB pigs (blue color) and genes predicted to be involved in enrichment of these functions. Genes upregulated in IB pigs are highlighted in green. Lines ending in arrow represent activation; lines ending in a bar represent inhibition. Blue lines indicate activation of the biological function. Yellow lines represent findings inconsistent with the state of the biological function and grey lines a non predicted effect.

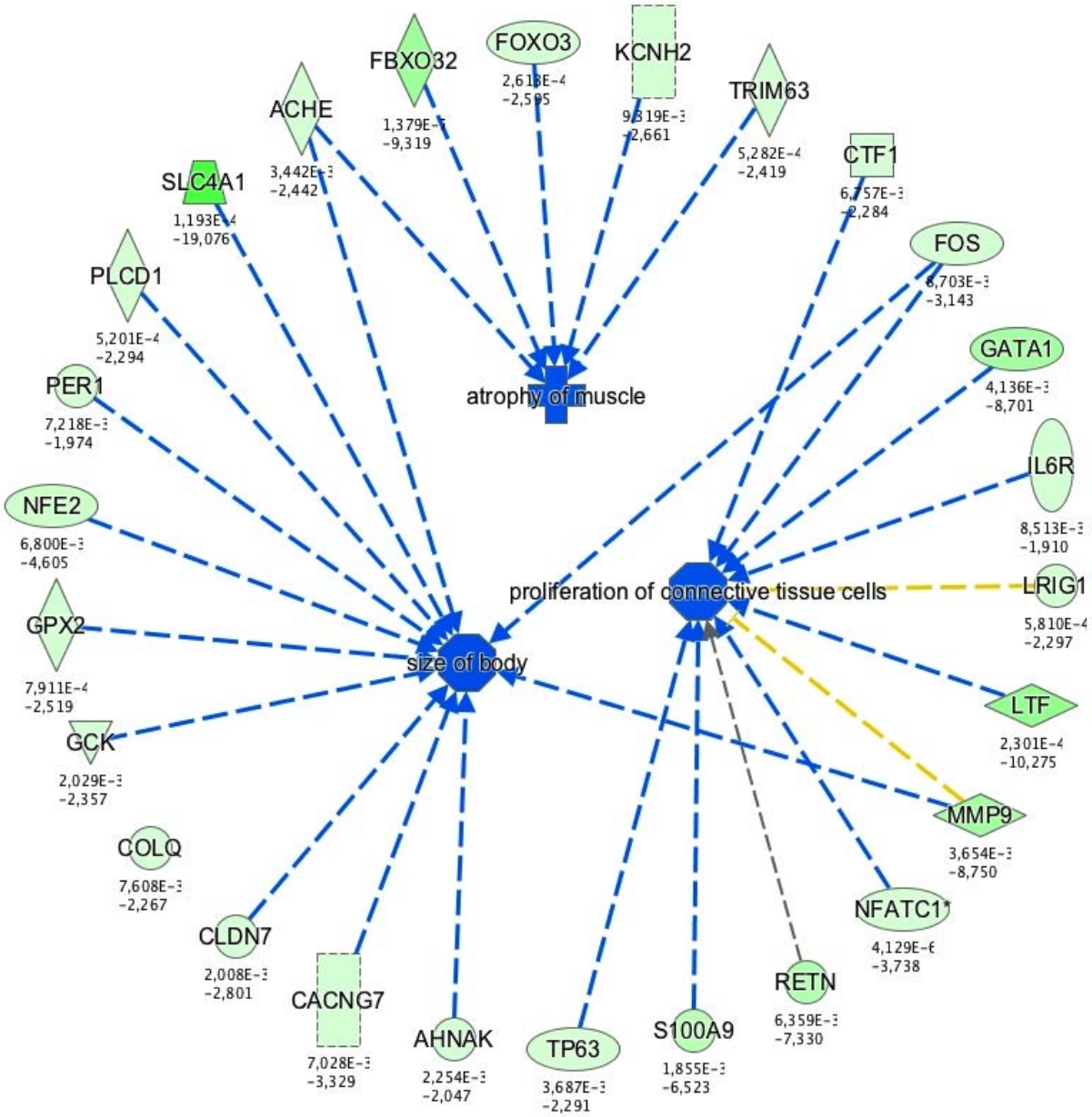


Muscle growth is not only determined by cell proliferation, but also by protein synthesis and degradation and angiogenesis. Protein degradation seems to be an active process in both IB and IBxDU newborn piglets. In the present study, IBxDU pigs showed upregulation of genes (*ELANE, MMP9, FBXO32, PVALB, HSPS1, HSPA4L* and *DNAJA1*) and pathways (Table 4) related to protein degradation, although enrichment of muscle degradation or atrophy functions was observed in IB

(Fig 2) but no in IBxDU pigs (Table 5), similarly to the results observed in BF muscle. This suggests greater muscle degradation in newborn IB than IBxDU pigs (Rivera-Ferre et al., 2005). Accordingly, genes showing high upregulation in four months-old IB pigs were mainly related to protein turnover and degradation (*CTSF, ADAMTS8* or *CELA2*).

Fig 2:Enriched biological functions related to growth and development in newborn IB piglets.

The network generated by IPA software shows enriched biological functions in IB pigs (blue color) and genes predicted to be involved in enrichment of these functions. Genes upregulated in IB pigs are highlighted in green. Lines ending in arrow represent activation; lines ending in a bar represent inhibition. Blue lines indicate activation of the biological function. Yellow lines represent findings inconsistent with the state of the biological function and grey lines a non predicted effect.



Energy homeostasis, inflammation and immune system

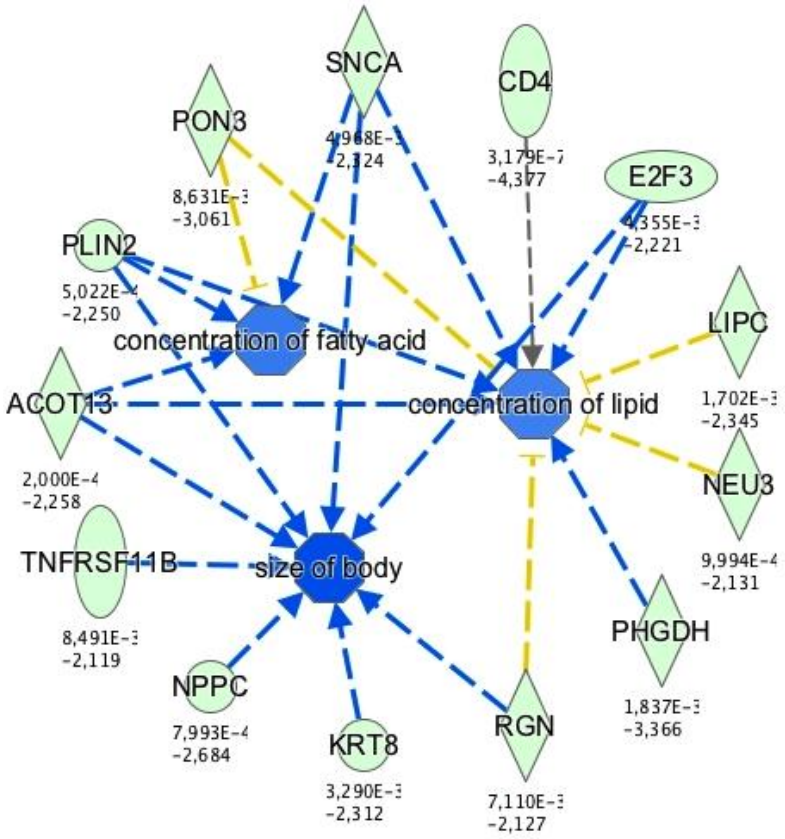
Energy homeostasis is closely related to other biological processes such as the immune system regulation, and has been specifically associated with inflammation mediators (Balasubramanyam, 2013; Exley et al., 2014). This connection might determine the enrichment of the immune system functions observed in both genetic types, with different genes and pathways implicated in each genotype (Firdous, 2014; Nishide et al., 2015; Rosc et al., 2015). However, results in this context are not clear and thus, interpretation of such results is difficult and may be speculative.

Energy homeostasis is tightly regulated in animals due to its importance for normal growth and survival. Pathways involved in cholesterol (*LXR/RXR Activation, Atherosclerosis Signaling*) and glucose metabolism (*Glucocorticoid Receptor Signaling, GDP-glucose Biosynthesis and Glucose* and *Glucose-1-phosphate Degradation*) were enriched in both newborn and four months old IB pigs, suggesting an increased energy metabolism in pure Iberian piglets. However the DE genes between genotypes involved in such pathways were different at birth and at four months of age, probably due to the high complexity of molecular mechanisms regulating lipid and glucose metabolism.

A pathway involved in the control of energy homeostasis, cell metabolism and muscle development, the *Wnt/Ca+ signaling pathway* (Sethi and Vidal-Puig, 2010) was enriched ($p = 0.009$) in newborn IB pigs. This pathway included genes upregulated in IB piglets, which were involved in adipogenesis and in the development of obesity, as *NFATC1* and *PLCD1* (Neal and Clipstone, 2003; Hirata et al., 2011). In agreement biological functions related to glucose metabolism and lipid accumulation, as *concentration of lipid, concentration of fatty acid* and *concentration of triacylglycerol* (Fig 3), were enriched in four months old IB pigs. Moreover, the *LPS/IL-1 Mediated Inhibition of RXR* pathway, enriched in four months old IB pigs was reported to positively correlate with fat area (Ponsuksili et al., 2011).

Fig 3: Enriched biological functions related to lipid metabolism in four months old IB pigs.

The network generated by IPA software shows enriched biological functions in IB pigs (blue color) and genes predicted to be involved in enrichment of these functions. Genes upregulated in IB pigs are highlighted in green. Lines ending in arrow represent activation; lines ending in a bar represent inhibition. Blue lines indicate activation of the biological function. Yellow lines represent findings inconsistent with the state of the biological function and grey lines a non predicted effect.



On the other hand, the *glucocorticoid receptor signaling* pathway, was enriched in both IBxDU and IB newborns, which might be related to the farrowing stress (in newborns) and with the wide range of actions associated to this pathway, from catabolic processes to glucose and energy homeostasis or adipocyte differentiation (Peckett et al., 2011; Kadmiel and Cidlowski, 2013). However, the upregulated genes in IBxDU pigs associated to this pathway are members of the HSPs family, and thus, its activation might be a consequence of cellular stress, or activated protein catabolism. On the other hand, DE genes involved in this pathway in IB pigs play important roles in protein catabolism (*FOXO3A*, (Jaitovich et al., 2015)), but also in lipid metabolism and adipogenesis (*NFATC1, FOS, FOXO3A*, (Lee et al., 1996; Neal and Clipstone, 2003; Wang et al., 2011)) and in osteogenesis and glucose uptake (*BGLAP*, (Villafán-Bernal et al., 2011)). Thus, although the *glucocorticoid receptor signaling* pathway was enriched in both IB and IBxDU piglets, a different set of DE genes was involved in each genetic type and thus, different metabolic consequences might be expected.

The juvenile IBxDU pigs showed enrichment of pathways mainly involved in biosynthesis and degradation of retinoids, such as retinoate, and retinol (active forms of vitamin A with known functions in immunity, reproduction, development and cell growth (Blaner, 1994)) or melatonin, an hormone involved in the synchronization of the circadian clock, associated with the control of energy homeostasis, among a wide range of functions (Fonken and Nelson, 2014). The enrichment of these functions is concordant with the upregulation of genes involved in degradation of compounds such as steroids and fatty acids (*CYP1A1*) (Liu et al., 2013). The *GABA receptor signaling* pathway was also found enriched in four months old IBxDU pigs. Beyond its function as an inhibitory neurotransmitter (Lujan et al., 2005), the expression of GABA receptor in human muscle was associated with increased resting energy expenditure (Wu et al., 2011), associated with energy balance. Thus, IBxDU pigs might have a greater basal energy expenditure that would decrease their potential for fat accumulation, in agreement with enriched biological functions and with the lower IMF content observed in IBxDU pigs.

Regulators analysis

We performed a regulatory factors study to investigate the driving molecular mechanisms responsible for the differences in gene expression observed between genetic types. Two different approaches, based on bibliographic (IPA software) and co-expression (RIFs analysis) information were combined as a powerful strategy to identify TRFs (Ayuso et al., 2015b). More DE genes and more TRFs were identified in younger than in older animals (261 vs 113 and 122 vs 62, respectively), suggesting a more different metabolism between genotypes and a more complex gene expression regulation in newborn pigs.

However, although differential regulation was expected, 16 TRFs were identified at both ages. These common TRF would be expected to have a more important role and a deeper impact in the final phenotype and thus, should be considered as strong candidate genes driving phenotypic differences in pure and crossbred Iberian pigs. Some of them, such as *MYOD1*, a well-known myogenic regulator, and some TRF recently associated with the regulation of *MYOD1* (*BHLHE40* and *HDAC2*; (Hsiao et al., 2009; Cho et al., 2015)) are necessary for muscle development. Similarly, *CTNNB1* is a component of the *Wnt signaling* pathway, related to cell differentiation and metabolism, including myogenesis and muscle regeneration in adult animals (Sethi and Vidal-Puig, 2010). Also, TRF involved in the immune response (*IRF9, NFKBIA, REL*) were found to affect gene expression in IB and IBxDU newborn and four months old pigs. *NFKBIA* has a critical role in the upregulation of pro-inflammatory factors and is considered a link between immunological stress and obesity (Zhang et al., 2008; Miller et al., 2010). Iberian pig accumulates more fat than other breeds and is considered as obese pig (Ovilo et al., 2014c). Thus, identifying TRF such as *NFKBIA*, *ATF4* or *CEBPA*, with direct roles in adipogenesis, and fat accumulation (Ren et al., 2014; Yu et al., 2014) is of high interest to understand regulatory mechanisms responsible for phenotypic differences.

Regarding the age-specific TRF identified, 12 regulator genes (*CREB3L1, CREBBP, FOXO1, HSF1, KLF1, MEF2C, MEF2D, MYOG, NFE2, SF1, SOX4* and *TEAD3*) were identified using the two approaches at birth. It is noteworthy that some of them (*MEF2C, MEF2D, MYOG, SOX4)* are important transcription factors involved in muscle cell differentiation (Edmondson et al., 1992; Jang et al., 2013). Moreover, TRF related to protein degradation (*CREB3L1, HSF1* and *CREBBP*) and to blood cells differentiation (*KLF1, SF1* and *NFE2*) were also identified and may play a role in muscle development, due to the need of protein degradation and blood supply on the growing muscle. Moreover, *KLF1* was strongly upregulated in IBxDU pigs (25.61), which suggest an important role of this TRF on differences between IB and IBxDU piglets. Another important TRF identified in the two performed analysis was *FOXO1*, which has been related to diverse processes that may affect phenotypic characteristics of IB and IBxDU piglets. *FOXO1* is involved in both, myoblast differentiation (Hakuno et al., 2011) and regulation of adipogenic genes (as *PPARG*) expression (Gupta et al., 2013). This TRF was also identified in BF muscle of newborn piglets (Ayuso et al., 2015b).

In four months old pigs, only 3 TRF were identified (*EN1, IRF2* and *TCF7L2*) using the two approaches. *EN1* and *IRF2* play important roles in regulating development and cell cycle. Specifically, *IRF2* interacts with the *VCAM-1* (suggested to play a role in the differentiation of skeletal muscle) gene promoter in muscle cells and is responsible for its transcriptional activation (Jesse et al., 1998). *EN1* and *TCF7L2* interact with the *Wnt signaling pathway* (Bachar-Dahan et al., 2006), which negatively regulates adipogenesis (Ross et al., 2000). Variants in this gene affect insulin secretion and body mass index and to promote type II diabetes (Florez et al., 2006; Munoz et al., 2006). Thus, it seems that lipid metabolism might be more tightly regulated in four months old IB and IBxDU pigs.

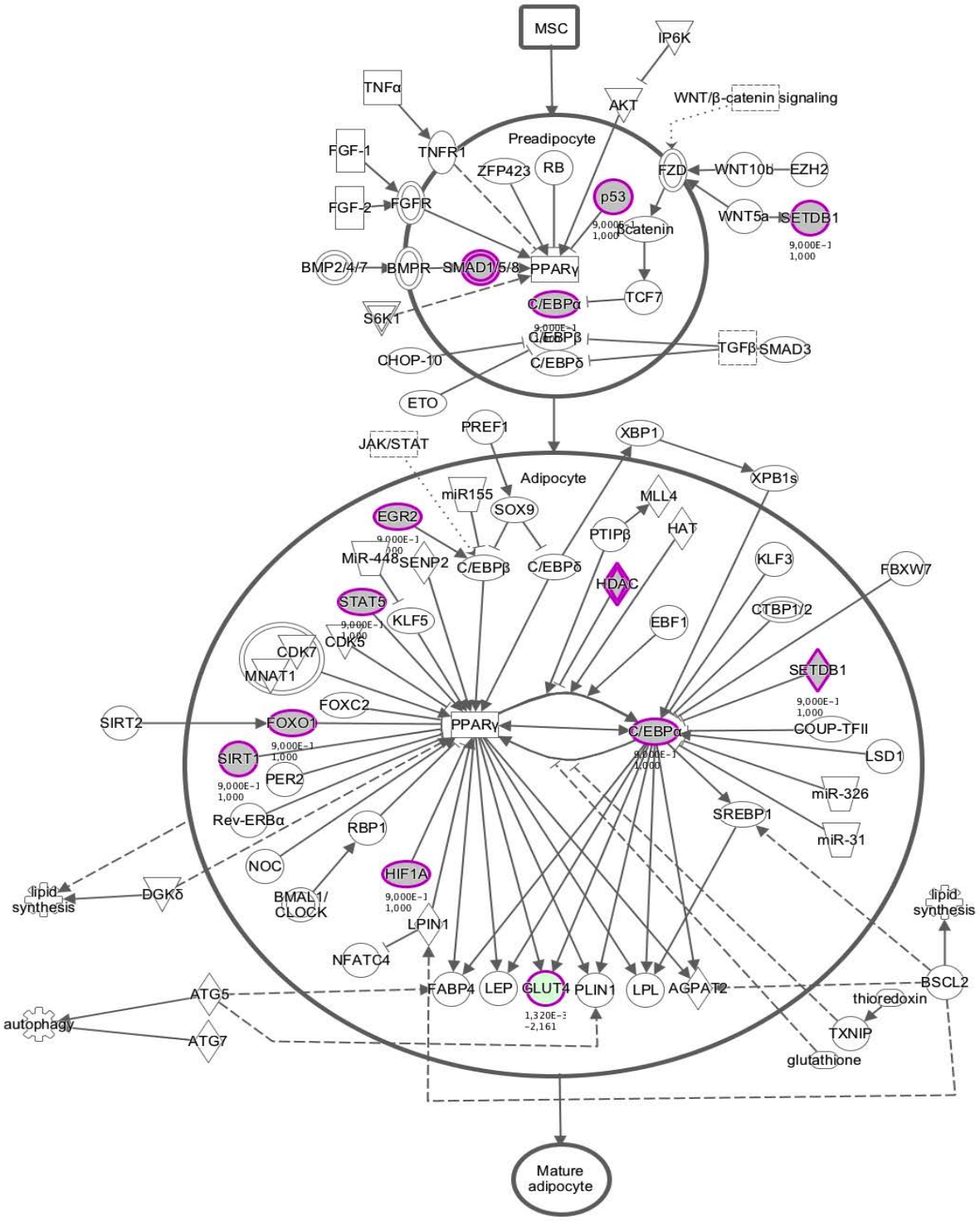
To better understand the role of the identified regulators on gene expression and in phenotypic differences, information from the DE and regulators analyses was used for biological interpretation, focusing on enriched metabolic pathways (Table S5). The *glucocorticoid receptor signaling* and the *adipogenesis* (Fig 4) were the most enriched pathways at birth. Changes in regulation and function of *adipogenesis* pathway in newborn piglets may determine the differences in fatness observed in LD muscle of IB and IBxDU pigs, in accordance with results observed in BF muscle of newborn piglets (Ayuso et al., 2015b). The *glucocorticoid receptor signaling* may be also implicated in such differences, due to its roles in energy homeostasis and adipocyte differentiation (Peckett et al., 2011; Kadmiel and Cidlowski, 2013).

The *aryl hydrocarbon receptor* pathway was the most enriched one in four months old pigs. This pathway is involved in xenobiotics metabolism, although a role in inhibition of lipid biosynthesis and adipocyte differentiation has also been reported (Alexander et al., 1998).

A total of fifty-one pathways were enriched at both stages, probably associated with processes relevant for animals' growth and development. Among them, the *PPAR signaling, the aryl*

hydrocarbon receptor, *the adipogenesis, the Wnt* and the *unfolded protein response* pathways, involved in adipocyte differentiation and protein degradation may be closely implicated in the phenotypic differences observed between IB and IBxDU pigs. Moreover, their identification along pigs' growth suggests a role in pure and crossbred Iberian adult phenotypes.

Fig 4: Adipogenesis pathway.
Genes upregulated in IB pigs are highlighted in green color by IPA software. Genes colored in grey are transcription factors.



Effect of muscular tissue, Biceps femoris or Longissimus dorsi, on gene expression

Longissimus dorsi and BF pig muscles have been previously reported to differ in phenotypic characteristics (Sharma et al., 1987; Kim et al., 2008), enzyme activity (Domínguez et al., 2014), gene expression (Sobol et al., 2015) and proteome profile (Te Pas et al., 2011), which might be caused by differences in metabolism associated to muscle fiber type (glycolytic vs oxidative) (Leseigneur-Meynier and Gandemer, 1991; Andrés et al., 2001). In the present study, differences in the transcriptomic profile were observed between BF and LD of newborn IB and IBxDU pigs. However the effect was much smaller than that reported when comparing LD and *Semimembranosus* muscles (Herault et al., 2014), probably due to the sampling at an early age, when muscle fiber type is not still determined (Ashmore et al., 1973; Picard et al., 2002).

In the present study, 83 genes were upregulated in LD muscle, some of them (*IBSP, ZIC1* and *MMP13*) showing large expression differences. *IBSP* is a structural protein of the bone matrix whereas *ZIC1*, a transcription factor important during development, might play a role in the early control of myogenesis (Mizugishi et al., 2004; Pan et al., 2011). Similarly, *MMP13* has been recently reported as a potential regulator involved in myostatin signaling and thus in muscle development and regeneration (Lei et al., 2013; Yang et al., 2015). On the other hand, genes highly upregulated in BF muscle are involved in myogenesis control (*HOXA11*), by regulating *MYOD* expression (Yamamoto and Kuroiwa, 2003), muscle contraction (*PVALB*), or the immune response and adipocyte differentiation, since *CXCL13* was found upregulated in adipocytes when compared to preadipocytes (Kabir et al., 2014). *PVALB* gene expression was also deeply affected by age (larger expression in four months old pigs) and by genetic type in newborn pigs, being upregulated in both BF and LD muscles of IBxDU pigs (Ayuso et al., 2015b). The finding of this DE gene across tissues, ages and genetic types suggests an active role on phenotypic changes.

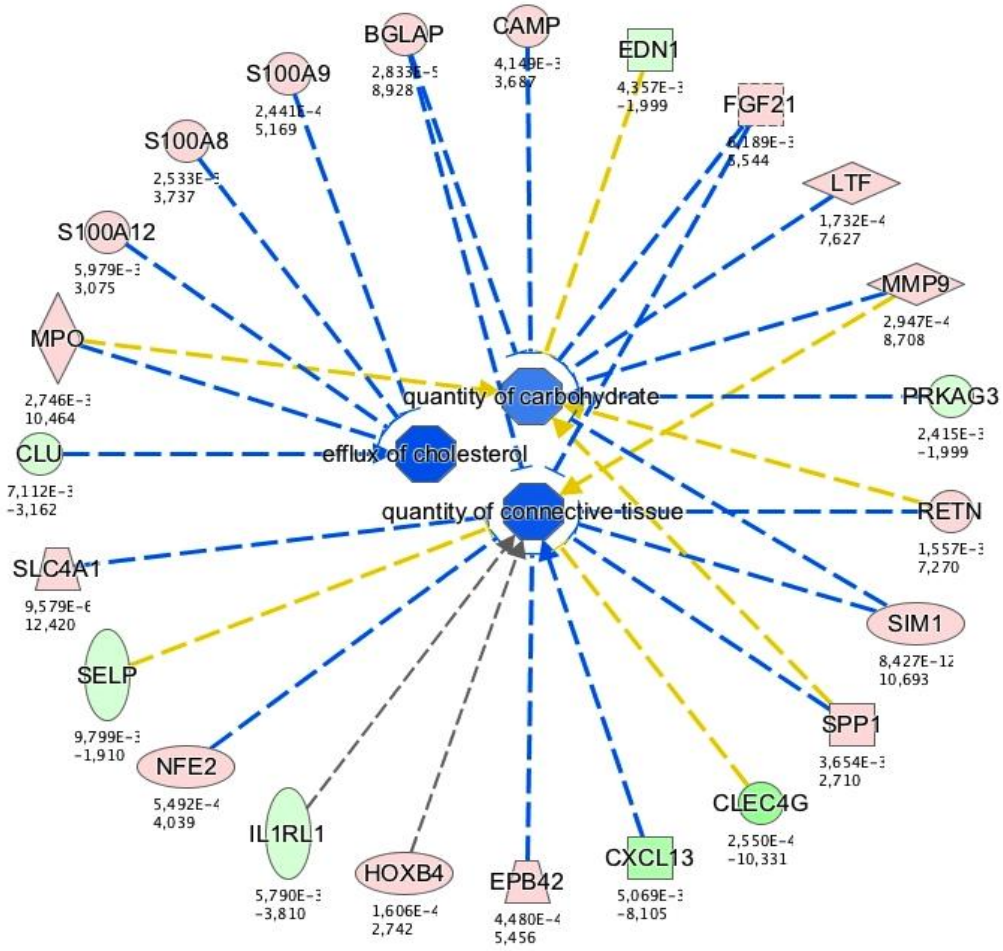
The biological interpretation of the DE analysis retrieved several enriched pathways in LD muscle related to lipid metabolism (*Atherosclerosis Signaling* and *VDR/RXR Activation*) muscle development and function (*Calcium Signaling, Inhibition of Matrix Metalloproteases* and *Actin Cytoskeleton Signaling*) and to the immune response (*Agranulocyte Adhesion and Diapedesis, Leukocyte Extravasation Signaling, Granulocyte Adhesion and Diapedesis* and *IL-8 Signaling*). In agreement with the enriched pathways, several biological functions related to inflammation and immune response (inflammation of organ, activation of mononuclear leukocytes, cell movement of monocytes and accumulation of leukocytes) were predicted by IPA software to be activated in LD muscle. On the other hand, functions such as *proliferation of cells, size of body, migration of cells* or *activation of connective tissue cells* are related to cell growth and development, being muscle development a process of special relevance in LD muscle of newborn pigs. In order to supply the needed oxygen and energy to growing cells, vascularization is required prior to muscle development. Both processes are closely related (Borselli et al., 2010; Garcia et al., 2013; Palstra et

al., 2014), and thus, the enrichment of biological functions (*neovascularization, development of cardiovascular system* and *vasculogenesis*) and the *HIF1α Signaling* pathway in LD muscle suggests a more active developmental stage in this muscle.

On the other hand, BF muscle showed enriched pathways involved in adipocyte differentiation and lipid metabolism (*LXR/RXR Activation, VDR/RXR Activation, Atherosclerosis Signaling*, *FXR/RXR Activation* and *biosynthesis of retinoids, bile acids and thyroid hormone*; (Bonet et al., 2003; Brandebourg and Hu, 2005; Brun et al., 2013; Xia et al., 2015)), in agreement with the enrichment of *efflux of cholesterol* in BF muscle. This suggests a more active lipid metabolism in BF muscle. In agreement, *quantity of connective tissue* and *quantity of carbohydrate* functions are also enriched in BF when compared to LD muscle (Fig 5), probably associated to a different energy homeostasis in both muscles. Thus, a more active lipid metabolism in BF muscle might allow a deeper impact of the genotype on lipid deposition, leading to the observed significant differences in IMF content between pig genotypes in BF, which are not evident in LD muscle of neonates. Moreover, these differences in lipid metabolism may be associated with the higher IMF content reported in BF than in LD muscle in adult pigs (Ayuso et al., 2015a). Pathways involved in biosynthesis and metabolism of numerous glycosaminoglycans were found enriched in BF muscle. Glycosaminoglycans are important constituents of the ECM that participate in the maintaining of appropriate extracellular conditions, required for myocyte differentiation (Osses and Brandan, 2002).

Fig 5: Enriched biological functions in *Biceps femoris* (BF) muscle.

The network generated by IPA software shows enriched biological functions in BF muscle (blue color) and genes predicted to be involved in enrichment of these functions. Genes upregulated in BF muscle are highlighted in green color and genes upregulated in *Longissimus dorsi* muscle are highlighted in red color. Lines ending in arrow represent activation; lines ending in a bar represent inhibition. Blue lines indicate activation of the biological function. Yellow lines represent findings inconsistent with the state of the biological function and grey lines a non-predicted effect.



Beyond metabolic pathways and biological functions, we used IPA software to identify potential regulators responsible for gene expression differences between BF and LD muscles. Among the identified regulators, 3 TRF (*HOXA10, HOXC8* and *SIM1*) were found DE between tissues and were then considered more consistent results. The homeobox family of TRF is involved in embryonic development and specifically in patterning all the musculoskeletal tissues of the limb (Izpisúa-Belmonte and Duboule, 1992). Moreover, in many studies *Hox* genes have been found as driving factors in stem cell differentiation towards adipogenesis (Cantile et al., 2003). *HOXA10* gene shows different expression patterns depending on the location of the adipose depot (Karastergiou et al., 2013) and *HOXC8* was reported as a lineage-specific adipocyte marker, showing higher expression in white fat depots than brown fat depots (Mori et al., 2012). *SIM1* gene is a TRF expressed during embryogenesis. It is mainly involved in hypothalamus development (Tolson et al., 2010), but is also expressed in early limb muscle precursor cells (Coumailleau and Duprez, 2009). Moreover, alterations in this gene have been widely associated with obesity development (Tolson et al., 2010;

Bonnefond et al., 2013). Although no information exists regarding *SIM1* gene effects on pigs, the DE across tissues, ages and genetic type in newborn piglets, together with existing information in other species, suggest this TRF as a strong candidate gene for fatness traits.

Limitations

Gene expression is dependent on temporal and anatomical location factors, both of them addressed in the present study. However, only two muscles and time points were considered due to the broad scope of the study. Longissimus dorsi and BF muscle represent the most important cuts within pork production, the loin and the ham. On the other hand, the two time points were selected due to the interest of comparing the initial (birth) and an intermediate (four months of age) stages during growing period, which in traditional Iberian pig production is considered up to 8 months of age (López-Bote, 1998).

Another limitation of the present study arises from phenotypic results regarding the age x genotype interactions, which suggest a similar interaction at the transcriptome level. However, complex models were not employed due to software limitations, because only one comparison can be studied at a time using CLC genomic workbench, and to the difficulty of interpretation of interaction effects in transcriptomic studies, which is out of the scope of the present work. The future reanalysis of the present data and results using flexible software that allow the examination of these interactions would be interesting.

3.2.6- Conclusions

The present study reports the effects of age, muscle and genetic type on phenotype, transcriptome, metabolic pathways and transcriptional regulation, associated with traits of interest. Age represented the most drastic influence on phenotype and transcriptome. Newborn pigs showed enrichment of anabolic functions, while predominant functions at four months of age were related to catabolism and muscle functioning, indicating a decrease in developmental and growth processes and a more advanced muscle development in juvenile pigs. Moreover, phenotypic differences regarding body size and plasma biochemical parameters were observed between genetic types at birth but disappeared at four months of age. This suggests strong differences in early growth patterns and metabolism between them, in spite of the closely related analyzed genotypes. In agreement, IMF differences between genotypes also depended on age and muscle. Gene expression results support the phenotypic findings, as DE genes and pathways suggest a different timing in growth, proliferative and anabolic processes. Those processes were upregulated in IBxDU newborn pigs (associated with a higher capacity for prenatal growth) and in four months old IB pigs (in agreement with a potential catch-up growth during the postnatal period).Differences

in metabolism were also observed, and results suggest a more active lipid and glucose metabolism in both newborn and four months old IB pigs, in agreement with their greater potential for fat accumulation. This is deducted from enriched pathways in IB (cholesterol and glucose-related and Wnt/Ca+ signaling pathways) and IBxDU pigs (GABA receptor signaling, associated with increased resting energy expenditure).An effect of muscle type on muscular metabolic characteristics was observed, as BF muscle showed increased lipid metabolism, while LD was characterized by growth and proliferative processes. The regulatory factors analysis retrieved several remarkable TRF (as *MYOD1, NFKBIA, FOXO1, MEFs, TCF7L2, SIM1* and *PVALB*), selected due to their identification following different methodological approaches, their identification across different ages and muscles and their potential roles on regulating molecular processes underlying differences in metabolism and productive traits between IB and IBxDU pigs.

Supporting Information

S1- Differentially expressed genes conditional on age (birth vs four months).

S2- Enriched pathways in the set of DE genes conditional on age (birth vs four months of age).

S3- Transcription factors affecting gene expression of *Longissimus dorsi* muscle from newborn and four months old Iberian pigs.

S4- Differentially expressed genes conditional on genetic type (Iberian (IB) vs Duroc X Iberian (IBxDU)) at birth and at four months of age.

S5- Transcription factors affecting gene expression of *Longissimus dorsi* muscle from pure and Duroc-crossbred Iberian pigs at birth and four months of age.

S6. Enriched pathways in the set of differentially expressed genes and transcription factors conditional on genetic type at birth and four months of age.

S7- Differentially expressed genes conditional on muscle (*Longissimus dorsi* (LD) vs *Biceps femoris* (BF)).

S8- Enriched pathways in the set of differentially expressed genes conditional on muscle type (*Longissimus dorsi* (LD) vs *Biceps femoris* (BF)) at birth and four months of age.

3.3- CAPITULO 3: Efectos de la restricción o suplementación de vitamina A y su período de aplicación sobre la acumulación de retinol y α-tocoferol y sobre la expresión génica en cerdos pesados

---

Effects of dietary vitamin A supplementation or restriction and its timing on retinol and α-tocopherol accumulation and gene expression in heavy pigs

Ayuso, M., Ovilo, C., Fernández, A., Nuñez, Y., Isabel, B., Daza, A., López-Bote, C.J., and Rey, A.I.

Anim Feed Sci Technol. 2015. 202: 62-74

Animal Feed Science and Technology 202 (2015) 62–74

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

Effects of dietary vitamin A supplementation or restriction and its timing on retinol and α-tocopherol accumulation and gene expression in heavy pigs

M. Ayuso[a,*], C. Óvilo[b], A. Fernández[b], Y. Nuñez[b], B. Isabel[a], A. Daza[c], C.J. López-Bote[a], A.I. Rey[a]

[a] Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain
[b] Departamento de Mejora Genética, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Ctra. Coruña Km. 7.5, 28040 Madrid, Spain
[c] Departamento de Producción Animal, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 10 October 2014
Received in revised form 13 January 2015
Accepted 14 January 2015

Keywords:
Dietary vitamin A
α-tocopherol
Gene expression
Dose-response
Heavy pigs

ABSTRACT

Vitamin supplementation is a widely extended practice in swine nutrition. Certain vitamins such as vitamins A and E are related to meat quality and have been reported as antagonists. Thus, their tissue levels are of interest for swine producers and consumers. This experiment was undertaken to study the effect of dietary vitamin A supplementation or withdrawal duration and timing on the evolution of vitamin A deposition in tissues, α-tocopherol accumulation and gene expression in heavy pigs. Eighty weaned Iberian piglets (16.3 ± 2.5 kg) were either fed a vitamin A-enriched diet (10,000 IU vitamin A/kg) (**CONTROL**) or given a diet without added vitamin A applied from the beginning of the trial at 16.3 kg (early restriction group, **ER**) or from an average weight of 35.8 kg (late restriction group, **LR**). Pigs fed ER and LR had lower ADG and worse feed efficiency than those from the CONTROL group at 101.4 kg (P=0.001 and P=0.034, respectively). However, final weight, average daily gain, average daily intake and feed conversion efficiency were not statistically affected by dietary treatment during the starter (<35.8 kg), fattening period (101.4–157.9 kg) or overall (16.3–157.9 kg). Retinol concentration in tissues reflected the dietary vitamin A level. Retinol and retinyl palmitate accumulation in hepatic and fat depots of control animals was more marked during the growing than during the finishing period. Retinol depots decreased in restricted groups and showed different sensitivity for mobilization between tissues, with faster retinol mobilization from the liver. The ER group had a higher hepatic α-tocopherol increase than the LR group (P<0.0001). However, in fat the increase in α-tocopherol levels were more marked in the LR than in the ER group (P<0.0001). *ADH1C* gene expression was higher (P=0.0237) in CONTROL than in ER at 101 kg and *LRAT* gene expression showed a dose-dependent decrease in the ER group at 101 and 158 kg LW (P<0.0001). There were no differences in *RBP4*, *ALDH1A1*, *MTTP* and *TTP* gene expression as affected by dietary treatment. Growth time influenced gene expression, with *ADH1C* and *RBP4* genes being mainly expressed at 101 kg LW compared to pigs at 36 or 158 kg LW (P<0.05). Relative expression of *MTTP* and *TTP* was also affected by time and showed an opposite pattern to that observed

Abbreviations: ER, early restriction group; LR, late restriction group; LW, live weight; ADG, average daily gain.

* Corresponding author. Tel.: +34 91 394 38 89; fax: +34 91 394 38 89.
E-mail address: mayuso@ucm.es (M. Ayuso).

http://dx.doi.org/10.1016/j.anifeedsci.2015.01.014

3.3.1- Abstract

Vitamin supplementation is a widely extended practice in swine nutrition. Certain vitamins such as vitamins A and E are related to meat quality and have been reported as antagonists. Thus, their tissue levels are of interest for swine producers and consumers. This experiment was undertaken to study the effect of dietary vitamin A supplementation or withdrawal duration and timing on the evolution of vitamin A deposition in tissues, α-tocopherol accumulation and gene expression in heavy pigs. Eighty weaned Iberian piglets (16.3 ± 2.5 kg) were either fed a vitamin A-enriched diet (10,000 IU vitamin A/kg) (**CONTROL**) or given a diet without added vitamin A applied from the beginning of the trial at 16.3 kg (early restriction group, **ER**) or from an average weight of 35.8 kg (late restriction group, **LR**). Pigs fed ER and LR had lower ADG and worse feed efficiency than those from the CONTROL group at 101.4 kg (P=0.001 and P=0.034, respectively). However, final weight, average daily gain, average daily intake and feed conversion efficiency were not statistically affected by dietary treatment during the starter (<35.8 kg), fattening period (101.4-157.9 kg) or overall (16.3-157.9 kg). Retinol concentration in tissues reflected the dietary vitamin A level. Retinol and retinyl palmitate accumulation in hepatic and fat depots of control animals were more marked during the growing than during the finishing period. Retinol depots decreased in restricted groups and showed different sensitivity for mobilization between tissues, with faster retinol mobilization from the liver. The ER group had a higher hepatic α-tocopherol increase than the LR group (P<0.0001). However, in fat the increase in α-tocopherol levels were more marked in the LR than in the ER group (P<0.0001). *ADH1C* gene expression was higher (P=0.0237) in CONTROL than in ER at 101 kg and *LRAT* gene expression showed a dose-dependent decrease in the ER group at 101 and 158 kg LW (P<0.0001). There were no differences in *RBP4*, *ALDH1A1*, *MTTP* and *TTP* gene expression as affected by dietary treatment. Growth time influenced gene expression, with *ADH1C* and *RBP4* genes being mainly expressed at 101 kg LW compared to pigs at 36 kg or 158 kg LW (P<0.05). Relative expression of *MTTP* and *TTP* was also affected by time and showed an opposite pattern to that observed for vitamin A-related genes. The results suggest that removing vitamin A from the diet for long or short periods in heavy pigs has the potential to reduce feed costs, increasing tissue α-tocopherol levels without affecting slaughter weight or feed efficiency.

Keywords: dietary vitamin A; α-tocopherol; gene expression; dose-response; heavy pigs.

3.3.2- Introduction

Vitamin A (retinol) is a liposoluble compound that essentially participates in reproduction, fetal development and cell metabolism (Gutierrez-Mazariegos, et al., 2011). Due to its important effects on metabolism, during recent years vitamin A has been included in diet formulation at higher doses than the minimum nutritional requirements established in pigs by NRC (2012) (D'Souza et al., 2003). Thus, commercial growing-finishing pig diets in the European Union contain vitamin A concentrations approximately six- to ten-fold higher than NRC recommendation (Fraga and Villamide, 2000).

Its action is mainly mediated by its active metabolite, all-trans-retinoic acid which activates transcription factors (Chambon, 1996; Chawla et al., 2001). Once inside the organism, most of the dietary vitamin A is stored in hepatocytes as retinol or mainly as retinyl esters, whereas the retinoic acid concentration is up to 1000-fold lower (Vogel et al., 1999). Retinol esterification is regulated by the enzyme lecithin:retinol acyltransferase (*LRAT*), which is highly responsive to exogenous retinoic acid (Ross and Zolfaghari, 2004). During periods in which dietary vitamin A is low or absent, retinyl esters stored in the liver are responsible for the maintenance of retinol levels (D'Ambrosio et al., 2011), retinol being mobilized from liver to extra-hepatic tissues by secretion of serum retinol binding protein (RBP4) (Alapatt et al., 2013).

In order to evaluate major sources of retinol for human supply, several studies have been carried out on retinol accumulation in pig tissues as affected by dietary vitamin A supplementation (Hoppe et al., 1992; Surles et al., 2007), with most of these studies being performed for a short period during animal growth. Moreover, the dietary vitamin A-depletion effect on retinol accumulation has been previously reported (Olivares et al., 2009b). Removing vitamin A from pig diet may result in enhancement of intramuscular fat and other metabolic alterations (Olivares et al., 2009a). Moreover, dietary vitamin A withdrawal may enhance vitamin E (α-tocopherol) accumulation (Olivares et al., 2009b), which has positive effects on meat quality characteristics (Dirinck et al., 1996) and is widely used to improve meat stability and shelf life due to its effects on lipid oxidation (Buckley et al., 1995; Rey et al., 2004). However, there is a lack of information on how retinol or retinyl esters are accumulated during pig growth and development and how a restriction period at different ages may affect vitamin A depots and vitamin E retention. Moreover, different studies have tried to clarify retinol metabolism and gene expression modulation in the organism (Ross and Zolfaghari, 2004). It has been found that long-term vitamin A status regulates *LRAT* enzyme activity and expression in the liver of rats (Zolfaghari and Ross, 2002). However, there is no information on how other enzyme expression such as alcohol dehydrogenase 1C (*ADH1C),* responsible for the oxidation of retinol to retinaldehyde in mice (Molotkov et al., 2003), or aldehyde dehydrogenase 1 (*ALDH1),* which irreversibly oxidizes retinaldehyde to retinoic acid (Kierfer et al., 2012), may be

affected by vitamin A-supplemented or vitamin A-restricted diets at different ages in the pig. Additionally, there is no information on the expression of these enzymes in heavy pigs, whereas it has been found that those involved in the vitamin A conversion to retinoic acid regulate fat in rodents (Kiefer et al., 2012).

The objectives of the present study were: 1) to quantify retinol accumulation in tissues (liver and subcutaneous fat) from heavy pigs fed a diet containing 7.5 x NRC vitamin A requirements [vitamin A requirements of pigs growing from 25 to 135 kg LW, 1,300 IU/kg diet (NRC, 2012)] during their whole growth and fattening periods and to study how it is mobilized when feeding a short-term or long-term vitamin A-restricted diet at different stages during growing; 2) to determine how these dietary treatments affect vitamin E accumulation in order to look for feeding strategies for improving tissue vitamin E levels; and 3) to explore how expression of genes involved in retinol and α-tocopherol metabolism and transport are affected by dietary treatment and growing period.

3.3.3 - Material and methods

Chemicals

All chemicals used were "HPLC" grade and were supplied by Panreac (Panreac Química S.A., Montcada i Reixac, Barcelona, Spain). Retinyl palmitate, retinol-all trans and α-tocopherol as pure standards were provided by Sigma Chemicals (Sigma Aldrich Química S.A., Alcobendas, Madrid, Spain). All reagents used for gene expression analysis were DNAse/RNAse-free.

Animals and Diets

Animal manipulations were performed in compliance with the regulations of the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used in experimentation. The experiment was specifically assessed and approved (report CEEA 2010/003) by the Spanish National Institute for Agricultural and Food Research and Technology (INIA) Committee of Ethics in Animal Research. The trial was conducted at CIA Dehesón del Encinar (Oropesa, Toledo, Spain).

Eighty castrated male (Torbiscal Pure Iberian) weaned piglets were housed in pens at a live weight (LW) of 11.7 (± 2.2) kg. At the age of 2 months with an average weight of 16.3 (± 2.5) kg piglets were randomly allotted into two groups of fifty and thirty piglets and housed in pairs. One group (n=50) was fed a vitamin A-enriched starter diet (10,000 IU vitamin A/kg diet) and the other group (n=30) received a starter diet formulated with no added vitamin A in the premix (early restriction group, **ER**). Two piglets, one from each group. At the age of four months and with an average weight of 35.8 (± 3.1) kg, nine piglets from the first group and nine piglets from the ER group were randomly selected and slaughtered. Also, twenty piglets from the first group started receiving the

vitamin A restricted diet (0 IU vitamin A/kg diet) (late restriction group, **LR**) and the remaining piglets (n=20) continued with the vitamin A supplemented diet (**CONTROL**). These levels of added vitamin A in the diet were maintained throughout the growing (from 35.8 to 101.4 kg LW) and finishing (from 101.4 to 157.9 kg LW) periods. During the finishing period, pigs were housed individually. Pigs were feed-restricted at 3.5%, 3% and 2.5% LW until four, eight and eleven months of age, respectively. Pigs had *ad libitum* access to water.

Performance parameters, such as live weight and average daily gain (ADG) were recorded at every growth stage and for all the experimental period.

Ingredients and chemical composition of experimental diets are shown in Table 1.

Table 1: Calculated analysis (g/kg, as-fed basis unless stated otherwise) of the experimental diets.

	Starter (<36 kg) [1]		Growth (36-101 kg) [2]		Fattening (101-158 kg)[3]	
	C[4]	R[5]	C	R	C	R
Calculated analysis[6]						
Net energy (MJ/kg)	10.0	10.0	9.5	9.5	10.4	10.4
Dry matter	895	895	990	990	990	990
Ash	48.0	48.0	49.2	49.2	48.9	48.9
Crude protein	178	178	158	158	135	135
Crude fat	41.9	41.9	26.8	26.8	82.3	82.3
Crude fiber	35.7	35.7	40.3	40.3	55.2	55.2
Vitamin A (retinyl acetate), IU/kg[7]	10,000	0	10,000	0	10,000	0
Vitamin E (α-tocopheryl acetate), mg/kg	26.7	26.7	26.7	26.7	26.7	26.7

[1, 2, 3] Ingredients (g/kg diet): (1) starter diet: Barley, 280; Soybean meal 440, 154.5; Wheat, 250; Corn, 192.9; Wheypowder, swet (cattle), 25; Lard, 16.6; Fullfat soybean toasted, 20; Calcium carbonate, 5.4; Dicalcium phosphate, 13.6; Mineral and vitamin premix, 4; Salt, 4; L-Lysine 50, 4; Methionine-OH, 1.4; L-Threonine, 0.6.
(2) growing diet: Barley, 500; Soybean meal 44, 169.4; Wheat, 290.3; Lard, 10; Calcium carbonate, 8.2; Dicalcium phosphate, 12; Mineral and vitamin premix, 4; Salt, 4.5; L-Lysine 50, 1.6. (3) fattening diet: Barley, 453.7; Soybean meal 44, 75.4; Wheat, 300; Lard, 20; High oleic sunflower seed, 120; Calcium carbonate, 8.2; Dicalcium phosphate, 4; Mineral and vitamin premix, 4; Salt, 4.5; L-Lysine 50, 2.2.

[4] CONTROL diet = Diet was enriched with 10,000 IU of vitamin A/kg as retinyl acetate

[5] RESTRICTED diet = Diet was formulated to contain 0 IU of vitamin A/kg as retinyl acetate

[6] According to Fundación Española Desarrollo Nutrición Animal (2010) (supplied per kg of diet).

[7] Vitamin A supplementation dose

Sample collection and chemical analysis

Different slaughters were carried out at 4, 8 and 11 months of age (Industrias Carnicas Alonso, S.L., Alcaudete de la Jara, Toledo, Spain) when pigs reached the average weights of 35.8 (±3.0) kg (n=18), 101.4 (±4.1) kg (n=30) and 157.9 (±7.0) kg (n=30). Samples of liver and adipose tissue at the level of the last rib were taken, weighed, vacuum-packed in low-oxygen permeable film and kept frozen at –20 ºC until analysis. Analyses were carried out over the course of the next 2 months. Samples for gene expression were immediately frozen in liquid nitrogen and kept under -80 °C until analysis.

Laboratory analysis

Retinol and α-tocopherol quantification

Concentrations of retinol, retinyl palmitate and α-tocopherol in liver samples were quantified according to the method described by Rey and Lopez-Bote (2001). Samples were homogenized in a 0.054 M dibasic sodium phosphate buffer adjusted to pH 7.0 with HCl. Absolute ethanol (3 ml) and hexane (2 ml) were added and mixed for 1 minute and the upper layer containing retinol and α-tocopherol was evaporated and dissolved in ethanol prior to analyses by reverse phase HPLC (HP 1100, with a diode array detector) (Hewlett Packard, Waldbronn, Germany). Separation was performed on a LiCrospher 100 RP-18 column (Agilent Technologies GmbH), the mobile phase was methanol:water (97:3 v/v) at a flow rate of 2 ml/min and the detector was fixed at 325 nm for the retinol and retinyl palmitate measurement (Olivares *et al.*, 2009 b) and at 292 nm for the detection of α-tocopherol.

Retinol and α-tocopherol in adipose tissue was analyzed using the method described by Rey *et al.*, (2006b). Samples (0.05 g) were saponified in the presence of KOH (50%), KCl (1.15%) and pyrogallol (3% in ethanol) at 70 °C for 30 min. Retinol and α-tocopherol were extracted in hexane, as previously described.

Gene expression quantification

Six genes were selected as candidate genes based on their roles on retinol or α-tocopherol metabolism. Selected genes were: retinol binding protein (*RBP4*), lecithin:retinol acyltransferase (*LRAT*), alcohol dehydrogenase 1 (*ADH1C*), retinaldehyde dehydrogenase 1 (*ALDH1*), microsomal triglyceride transfer protein (*MTTP*) and alpha-tocopherol transfer protein (*TTP*). Gene expression analysis was performed in hepatic tissue from ER and CONTROL groups at three different ages (4, 8 and 11 months). Total RNA was extracted from frozen liver samples using RiboPure RNA isolation kit according to the manufacturer's instructions (Ambion, Austin, TX, USA). The total RNA concentration was quantified using a NanoDrop-100 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA) and the RNA quality (RNA Integrity Number) was evaluated by an Agilent

2100 Bioanalyzer device (Agilent Technologies, Santa Clara, CA, USA). RNA Integrity Number (RIN) values obtained were in the range of 7.9-8.4, assuring the homogeneity and high quality of the samples. First-strand cDNA synthesis was performed using the Superscript II enzyme (Invitrogen, Life Technologies, Paisley, UK) with random hexamers in a total volume of 20 µl containing 1 µg of total RNA as template, following supplier instructions. Primers for amplification were designed using Primer Select software (DNASTAR, Wisconsin, USA) from the available GENBANK and/or ENSEMBL pig sequences, covering different exons in order to assure the amplification of the cDNA (Table 2).

Standard PCRs on cDNA were carried out to verify amplicon sizes. Transcript quantification was performed using SYBR Green mix (Roche, Basel, Switzerland) in a LyghtCycler 480 (Roche, Basel, Switzerland). The qPCR reactions were prepared in a total volume of 20 µl containing 2.5 µl of cDNA (1/20 dilution), 10 µl of SYBR Green mix and 0.15 µM of both forward and reverse primers. All real time qPCR reactions were performed in 384-well reaction plates (Roche, Basel, Switzerland). The thermal cycling conditions were 95 °C for 10 min., followed by 45 cycles of 95 °C (15 sec.) and 60 °C (1 min.) where the fluorescence was acquired. Finally, a dissociation curve to test PCR specificity was generated by one cycle at 95 ºC (15s) followed by 60 ºC (20s) and ramp up to 95 °C with acquired fluorescence during the ramp at 0.01 °C/s. Absence of contamination was ensured using a negative control, with water instead of cDNA template. Data were analyzed with LyghtCycler 480 SW1.5 software (Roche, Basel, Switzerland). All points and samples were run in triplicate as technical replicates and dissociation curves were carried out for each individual replicate. Single peaks in the dissociation curves confirmed the specific amplification of the genes. PCR efficiency was estimated by standard curve calculation using four points of cDNA 5-fold dilutions from a pool of samples. Values of PCR efficiency are indicated in Table 2 (ranged from 86.5 to 98.05). C_p values were employed for the statistical analyses of differential expression. Four commonly used housekeeping genes (*ACTB, B2M, GAPDH and TBP*) were tested with geNorm software (Vandesompele et al., 2002) to evaluate their stability. *GAPDH* and *ACTB* were selected.

Table 2: Primer design for qPCR, gene details and PCR efficiencies (eff, %) in hepatic tissue (L)

Gene name	Gene symbol	GenBank Acc. number	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Size (bp)	Eff (%)
Alcohol dehydrogenase 1C (class I)	*ADH1C*	NM001243939	ACCCCCGAAGGCCTATGAAGT	CTGGGGAACAAAGAGTGGGATGA	202	94.9
Aldehyde dehydrogenase 1 family, member A1	*ALDH1A1*	NM000689	CAGTGAAGGCCGCAAGACAAG	TGCAGCCTCCTAAATCCATCAGA	192	98.05
Lecithin retinol acyltransferase (phosphatidylcholine-retinol O-acyltransferase)	*LRAT*	NM001244920	CTCAAGAAGAAGGCGCTGCTCAA	ATTATCTTCACATTCTCACAAAA	173	86.5
Retinol binding protein 4	*RBP4*	NM214057	GCCGAGTGAGCAGCTTCCGAGTC	TGCGCACACGTCCCAGTTATTT	203	94.15
Microsomal Triglyceride Transfer Protein	*MTTP*	NM214185	CCGAAATCAGGTCTTGGGTGTCA	AGTCTGGGGCCTGCTTCTGTTG	192	98.01
Alpha-Tocopherol Transfer Protein	*TTPA*	XM005663062	AGACCTCCACCCTCGAAGCATCCT	TTCCGTTGTGTTTCTACCTCCTGT	201	93.85

Statistical analysis

The data were analysed as a completely randomized design using the general linear model (GLM) procedure contained in SAS version 9.2 (1999). Linear and quadratic patterns of the regression equations were carried out in order to estimate the relationship between kg of LW (independent variable) and vitamin A accumulated in tissues (dependent variable). The relationship between retinol and α-tocopherol was also quantified by regression equations. A Student's T test was used to compare slopes of the regression equations. For statistical analysis of performance parameters, initial weight was used as covariate when it was significant and removed from the model when not. The animal was the experimental unit for all data analysis. The results were considered to be significant at P<0.05.

Statistical analysis of gene expression data was carried out following the method proposed by Steibel et al. (2009), which consists of the analysis of cycles to threshold values (C_p), for the targets and endogenous genes using a linear mixed model. The following model was used for analyzing the joint expression of the target and control genes in different tissues:

$$y_{gijk} = TG_{gi} + L_{gj} + B_{gjk} + D_{ijk} + e_{gijk}$$

where $y_{gijk} = -log_2\left(E_{gijk}^{-Cp_{gijk}}\right) y_{gijk} = log_2\left(E_{gijk}^{-C_{gijk}}\right)$, E brings the efficiency of the PCR of each gene, C_p is the value obtained from the thermocycler software from each replicate of the gth gene in a sample collected from the kth animal fed with the ith dietary treatment, TG_{gi} is the specific effect of the ith dietary treatment on the expression of gene g, L_{gj} and B_{gjk} are specific random effects of the jth full-sib family and the kth pig on the expression of gene g, D_{ijk} is a random sample-specific effect common to all the genes, and e_{gijk} is a residual effect.

To test differences in the expression rate of genes of interest ($diff_{TG}$) between treatments and slaughter weights normalized by the endogenous genes, different comparisons were performed between the respective estimates of TG levels. Significance of $diff_{TG}$ estimates was determined with the t statistic. To obtain fold change (FC) values from the estimated $diff_{TG}$ values, the following equation was applied: $FC = 2^{-diff_{TG}}$.$\mathbf{FC = 2^{-diff_{TG}}}$ Asymmetric 95% confidence intervals (CI) were calculated for each FC value by using the standard error (SE) of the estimated difference: 95% CI from $2^{\left[-\left(diff\right]_{TG}+1.96\ x\ SE\right)}\mathbf{FC_{UP} = 2^{[-(diff]_{TG}-1.96\ x\ SE)}}$ $2^{(-\mathrm{diff_{TG}}+1.96\mathrm{xSE})}$.

3.3.4- Results

Pig growth performance

Performance parameters are shown in Table 3. Pigs on the vitamin A restricted treatments (ER and LR) grew significantly slower (P= 0.001) and used feed significantly less efficiently (P= 0.034) than CONTROL animals during the growing period (35.8-101.4 kg). However, final weight, average daily gain, average daily intake and feed conversion ratio were not statistically affected by dietary treatment during the starter (<35.8 kg LW) or fattening periods (101.4-157.9 kg LW). Overall performance was similarly unaffected by dietary vitamin A level.

Table 3: Effects of dietary Vitamin A enrichment (10,000 IU-CONTROL) and early (ER) or late restriction (LR) on the performance of pigs during the starter (from 16.3 to 35.8; 2-4 months of age), growing (from 35.8 to 101.4; 4-8 months of age) and finishing (from 101.4 to 157.9; 8-11 months of age) periods.

	TREATMENT EFFECT					
	CONTROL [1]	ER [2]	LR [3]	SEM	P value	P value covariate [4]
Initial weight, kg	16.1	16.5		0.42	0.48	
Final weight, kg (starter) [5]	35.9	35.7		0.34	0.58	<0.001
Average daily gain, kg (starter)	0.29	0.28		0.005	0.52	0.30
Feed average daily intake, kg (starter)	0.91	0.91		0.006	0.58	<0.001
Feed conversion ratio, kg gain/kg (starter)	3.22	3.26		0.004	0.53	<0.001
Final weight, kg (growing) [6]	103.2 [a]	100.1 [b]	100.6 [b]	0.76	0.01	<0.001
Average daily gain, kg (growing)	0.50 [a]	0.47 [b]	0.47 [b]	0.005	0.001	0.69
Feed average daily intake, kg (growing)	2.08	2.03	2.05	0.02	0.15	<0.001
Feed conversion ratio, kg gain/kg (growing)	4.18 [b]	4.31 [a]	4.32 [a]	0.004	0.03	<0.001
Final weight, kg (fattening) [7]	159.5	158.2	155.4	2.10	0.42	0.01
Average daily gain, kg (fattening)	0.81	0.82	0.79	0.03	0.75	0.37
Feed average daily intake, kg (fattening)	3.28	3.24	3.19	0.03	0.260	0.001
Feed conversion ratio, kg gain/kg (fattening)	4.05	3.99	4.11	0.13	0.85	0.94
Average daily gain, kg (total) [8]	0.52	0.51	0.50	0.008	0.36	0.47
Feed average daily intake, kg (total)	2.07	2.07	2.05	0.02	0.80	<0.001
Feed conversion ratio, kg gain/kg (total)	4.00	4.02	4.08	0.005	0.50	<0.001

[1] CONTROL: Pigs fed a vitamin A-enriched diet (10,000 IU/kg diet); [2] ER: Pigs fed a diet without vitamin A from 2 months of age (16.2 kg)
[3] LR: Pigs fed a diet without vitamin A from 4 months of age (35.8 kg); [4] P value covariate: P value of initial weight (covariate)
[5] Period from 2-4 months of age (starter): Control n= 45; ER n= 29; [6] Period from 4-8 months of age (growing): Control n= 20; ER n= 20; LR n= 16.
[7] Period from 8- 11 months of age (fattening); Control n= 10; ER n= 10; LR n= 8.
[8] Period from 2-11 months of age: Control n= 10; ER n= 10; LR n= 8
[a,b] Different superscripts in a column indicate significant difference (P<0,05)

Retinol accumulation in pig tissues

Dietary vitamin A was differently stored in pigs according to the tissue. In the first sampling (at 35.8 kg LW after 2 months of the dietary treatment) concentrations of fat and liver retinol in those pigs fed a 7.5xNRC vitamin A-supplemented diet (CONTROL) were 0.39 μg/g (± 0.05) and 6.98 μg/g (± 2.17), respectively (Figure 1A, 1B). Vitamin A supplementation also resulted in 10-fold higher hepatic storage of retinyl palmitate (67.8 μg/g ± 22.7) than retinol at the initial sampling time (Figure 1C). Retinol concentration in all tissues reflected dietary vitamin A supplementation. Hence, retinol depots increased over time in those pigs fed a vitamin A-supplemented diet, whilst they decreased in ER and LR groups (Figures 1A, 1B). The effect of dietary vitamin A restriction was clearly observed at 35.8 kg LW (after 2 months of vitamin A withdrawal), when the CONTROL group showed higher levels of retinol in fat and liver than the ER group. Moreover, this difference increased over time. As shown in Figure 1, a linear relationship was found between retinol concentration in fat or liver and slaughter weight that was expressed by the regression equations that appear in Table 4. In adipose tissue, CONTROL pigs increased retinol storage by 1.4 fold from 36 to 158 kg LW (R^2=0.66; P<0.0001), this increase being slightly higher during the growing than during the fattening period (1.3-fold vs. 1.0-fold, respectively). However, fat retinol depots linearly decreased with time by 4-fold in the ER group (R^2=0.88; P<0.0001). The negative slope in fat retinol depots was not affected by treatment length (ER vs. LR). In liver, CONTROL pigs showed a higher increase in retinol depots over time (5-fold) in spite of the greater variability observed in the fattening period. On the other hand, vitamin A-restricted pigs (ER group) suffered an 8-fold retinol decrease from the initial level. As observed in adipose tissue, liver retinol accumulation was more pronounced during the growing period than fattening (4-fold vs. 1.2-fold, respectively) (R^2=0.61; P<0.0001).

Figure 1.- Retinol accumulation (µg/g) in fat (A) and liver (B) and retinyl palmitate accumulation (µg/g) in liver (C) from pigs fed a vitamin A-enriched diet (10,000 IU/kg diet) (CONTROL) or vitamin A-restricted diet (0 IU/kg diet) at the early (ER) or late (LR) growing stage.

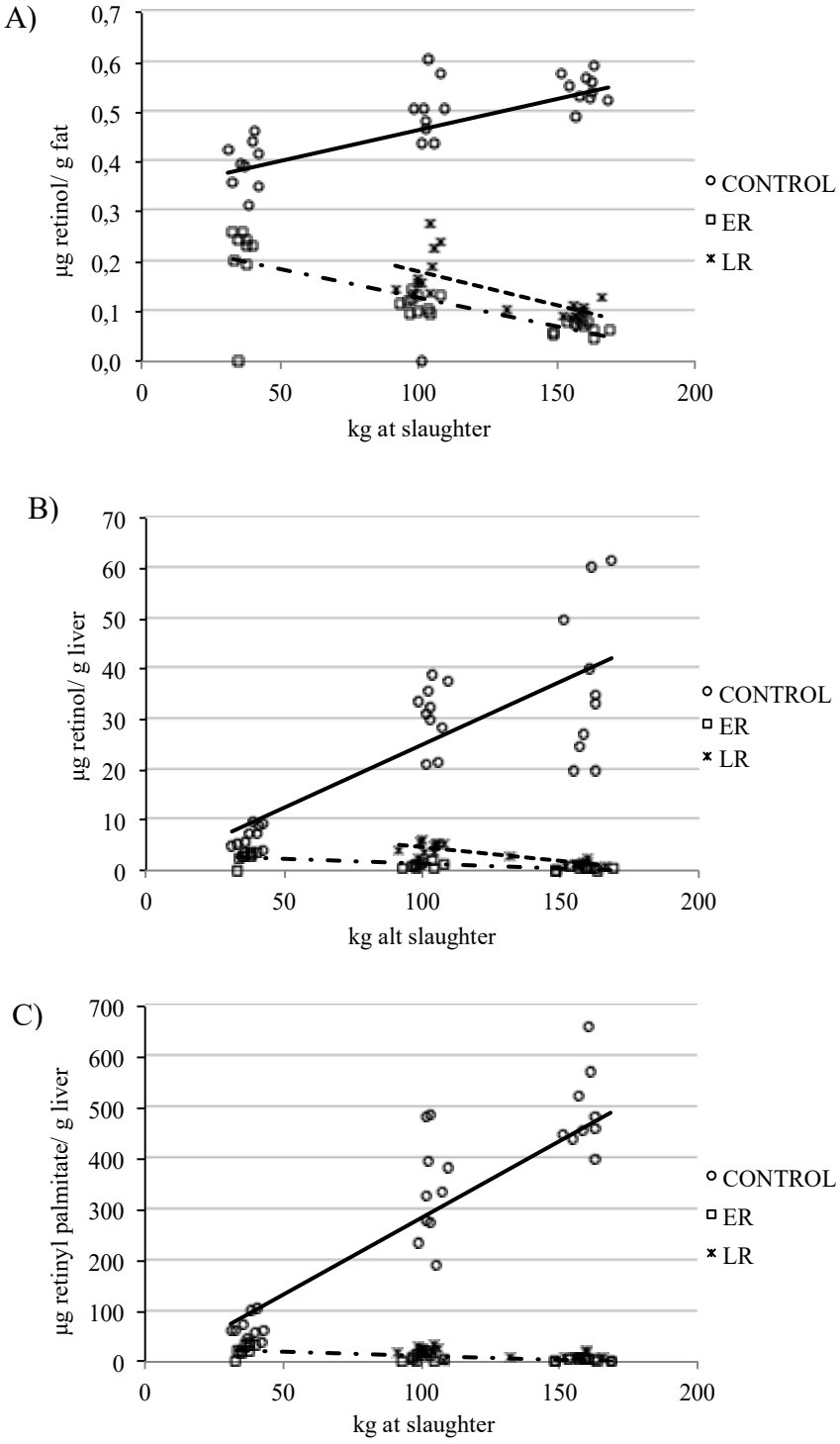


Table 4: Regression equations for fat and liver retinol (µg/g) and liver retinyl palmitate (µg/g) accumulation as a function of live weight (kg) and alpha tocopherol relationship with retinol in fat and liver tissue in pigs fed a Vitamin A enriched diet (10,000 IU/kg diet) and a Vitamin A restricted diet (0 IU/kg diet) imposed in the early (ER) or late (LR) growing stages.

	Treatment	N	Intercept		kg (x)		R^2	RSD	P (lineal)
Fat retinol	CONTROL[1]	28	0.36 ± 0.02		0.001 ± 0.0002		0.66	0.05	<0.001
	ER[2]	28	0.28 ± 0.01		-0.001 ± 0.0001		0.89	0.02	<0.001
	LR[3]	20	0.24 ± 0.02		-0.001 ± 0.0002		0.68	0.02	<0.001
Liver retinol	CONTROL	29	0.01 ± 4.37		0.25 ± 0.04		0.61	10.2	<0.001
	ER	28	3.68 ± 0.26		-0.02 ± 0.002	a	0.78	0.59	<0.001
	LR	20	10.9 ± 0.93		-0.06 ± 0.007	b	0.81	0.84	<0.001
Liver retinyl palmitate	CONTROL	28	-51.9 ± 33.4		3.53 ± 0.30		0.84	77.4	<0.001
	ER	28	31.3 ± 2.93		-0.19 ± 0.03		0.67	6.65	<0.001
	LR	20	43.9 ± 5.52		-0.20 ± 0.04		0.58	5.08	<0.001
	Treatment	N	Intercept		Tissue retinol (x)		R^2	RSD	P (lineal)
Fat α-tocopherol	CONTROL	29	6.57 ± 1.97		7.30 ± 3.96		0.11	1.73	0.08
	ER	29	15.7 ± 1.00		-22.4 ± 6.30	a	0.32	2.57	0.001
	LR	20	20.0 ± 2.22		-48.4 ± 14.6	b	0.38	3.51	0.004
Liver α-tocopherol	CONTROL	29	0.05 ± 2.50		0.44 ± 0.08		0.51	7.08	<0.001
	ER	29	22.2 ± 1.95		-5.71 ± 0.91	a	0.59	7.08	<0.001
	LR	20	24.3 ± 1.46		-2.88 ± 0.41	b	0.73	3.28	<0.001

[1]CONTROL group: Animals were fed a diet enriched with 10,000 IU of vitamin A/kg as retinyl acetate for their whole live.
[2]ER group: Animals were fed a diet with 0 IU of vitamin A/kg as retinyl acetate from two months of age (from 16.3 to 157.9 kg).
[3]LR group: Animals were fed a diet with 0 IU of vitamin A/kg as retinyl acetate from four months of age (from 35.8 to 157.9 kg).
[a,b] Different superscripts in a column indicate significant difference (P<0,05)

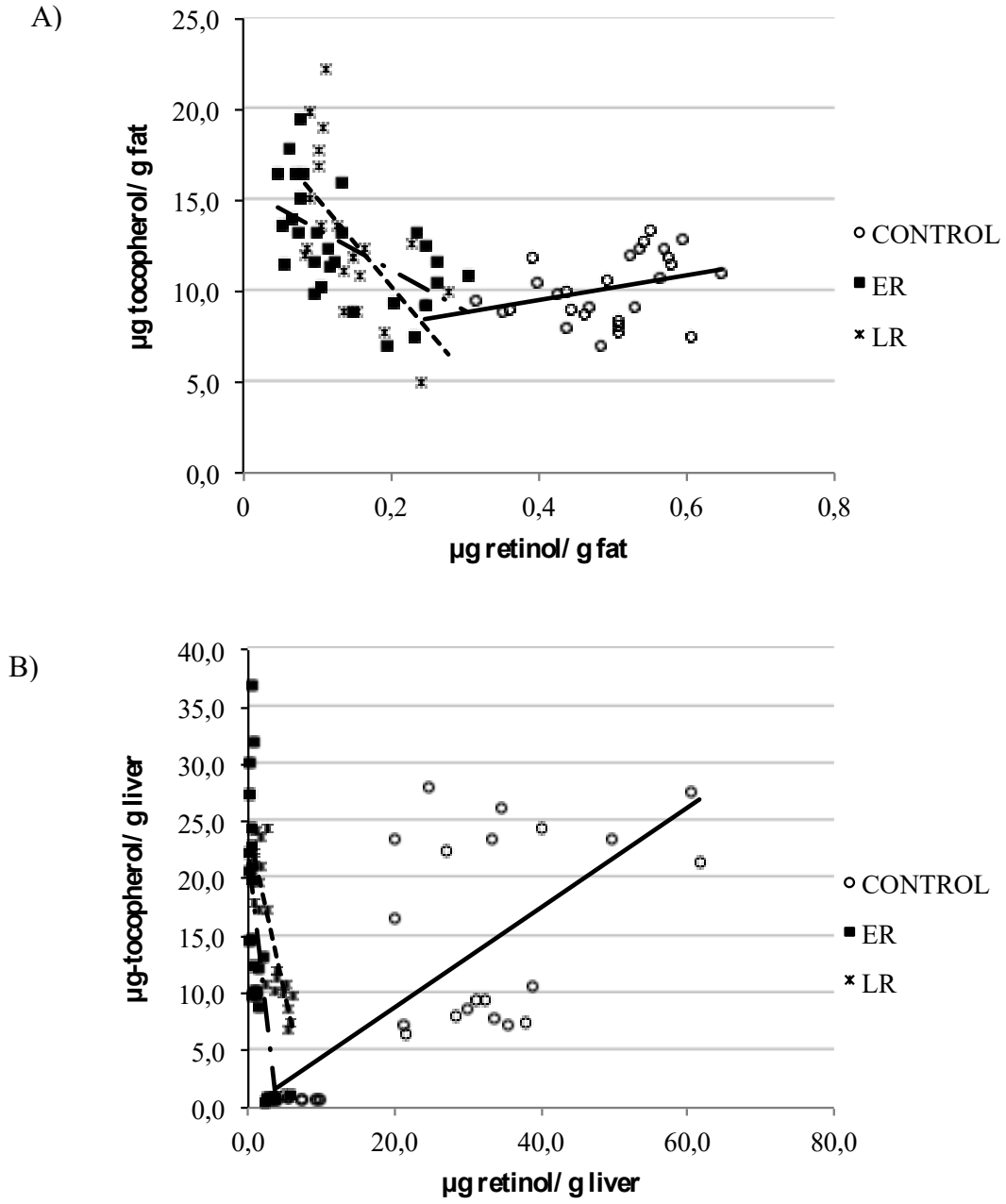
Moreover, liver retinol mobilization in the vitamin A-restricted groups was more marked in LR than in ER groups (3-fold vs. 2.5-fold) ($P<0.05$), with both groups reaching similar retinol levels at 158 kg LW (1.54±0.65 for LR group and 0.42±0.22 for ER group).

Figure 1C shows the change in liver retinyl palmitate depots during the evaluated period. Similar results as those observed for liver retinol depots were found. Retinyl palmitate levels in the CONTROL group increased almost 7-fold from 36 to 158 kg LW. Retinol mobilization from retinyl palmitate reservoirs in liver was similar to that observed for retinol storage in this tissue, without differences between the ER and LR groups either at 101 kg LW (8.56±5.84 and 24.43±6.42, respectively; $P > 0.05$) or at 158 kg LW (3.61±1.8 and 12.26±4.23, respectively; $P > 0.05$).

α-Tocopherol accumulation

The α-tocopherol concentrations in fat and liver as affected by vitamin A accumulation are presented in Figure 2. An inverse relationship between α-tocopherol and retinol concentrations was observed. The long term vitamin A-restricted pigs (ER) had a higher increase in hepatic α-tocopherol concentration than those short term-restricted (LR) ($P<0.0001$). However, in fat, α-tocopherol increase was more marked in the LR than in ER group ($P<0.0001$), as observed in the slope of the regression equations (Table 4). Concentrations of fat retinol < 0.2 μg/g resulted in higher α-tocopherol accumulation in fat.

Figure 2.- Fat (A) or hepatic α-tocopherol (B) concentration related to the tissue retinol level from pigs fed a vitamin A-enriched diet (10,000 IU/kg diet) (CONTROL) or vitamin A-restricted diet (0 IU/kg diet) at the early (ER) or late (LR) growing stage.



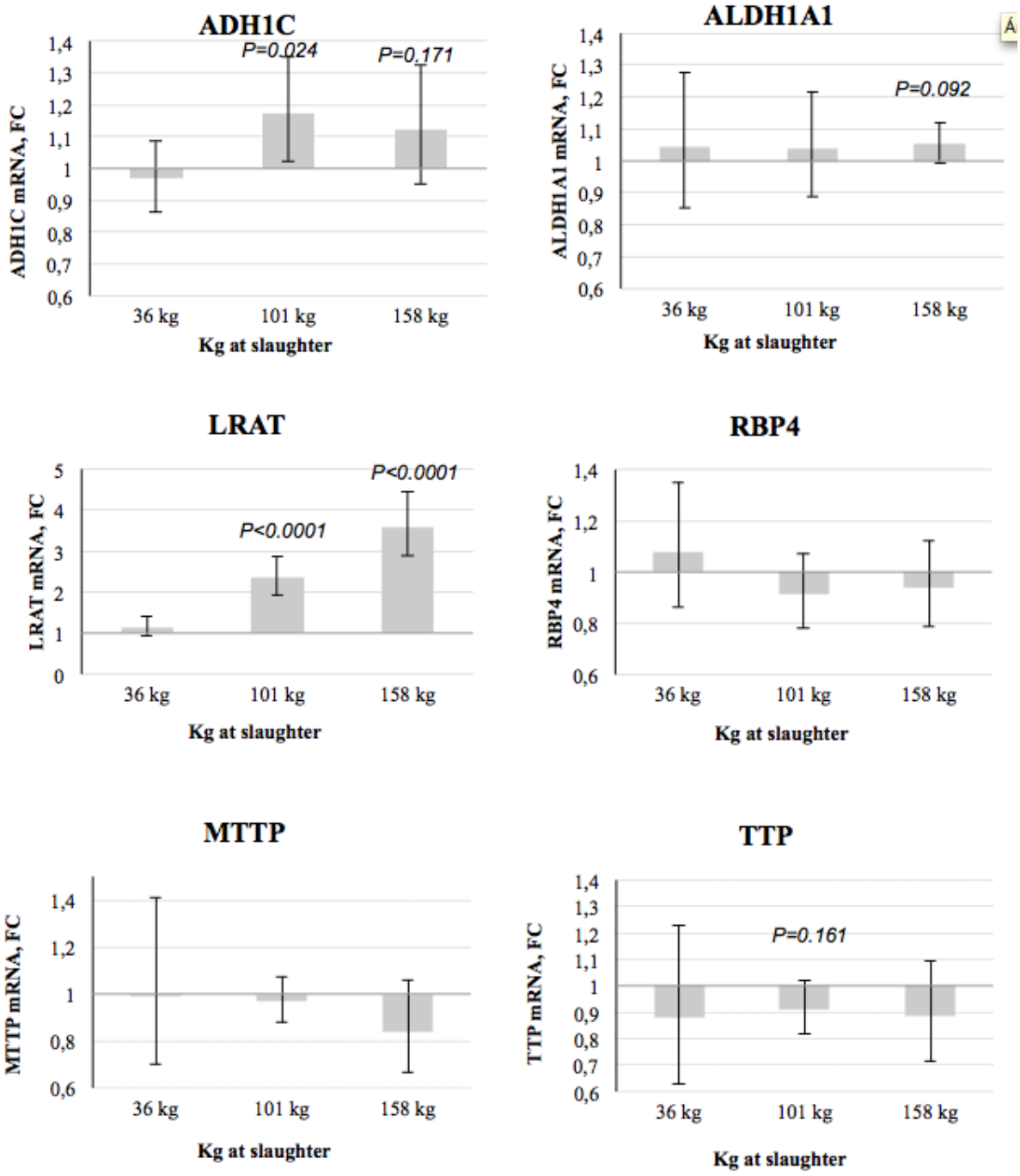
Expression of retinol and α-tocopherol metabolism-related genes as affected by dietary treatment

Four genes involved in vitamin A metabolism (*ADH1C, ALDH1A1* and *LRAT*) and transport (*RBP4*) were assessed in hepatic tissue from CONTROL and ER pigs at the three different slaughter weights (Figure 3). Differences in the *ADH1C* and *LRAT* genes expression among treatments were observed at 101 kg LW. CONTROL pigs showed higher expression levels of *ADH1C* ($P = 0.0237$) and *LRAT* ($P<0.0001$) than those fed a vitamin A-restricted diet (ER). Moreover *LRAT* gene expression was

higher in the CONTROL than ER group at 158 kg LW (P<0.0001), whereas *ADH1C* and *ALDH1A1* genes expression was also numerically higher in CONTROL although differences were not statistically significant (P=0.17 and P=0.092, respectively). However, no effects were observed according to dietary treatment at 36 kg LW for any of these genes or for *RBP4* in all the experimental period.

On the other hand, the expression of genes that participated in α-tocopherol metabolism and transport such as microsomal triglyceride transfer protein (*MTTP*) and alpha-tocopherol transfer protein (*TTP*) was not statistically affected by dietary treatment (Figure 3). However, we observed numerically higher means in the ER compared to the CONTROL group at 158 kg for *MTTP* (P=0.1433) and at 101 kg for *TTP* (P=0.1611).

Figure 3.- Relative expression (Fold Change, FC) of *ADH1C*, *ALDH1A1*, *LRAT*, *RBP4*, *MTTP* and *TTP* genes in liver from pigs fed a vitamin A-enriched diet (10,000 IU/kg diet) (CONTROL) or vitamin A-restricted diet (0 IU/kg diet) from two months of age (ER) at the end of the growing stage (101 kg). Fold change values>1 means greater expression in CONTROL than ER group.

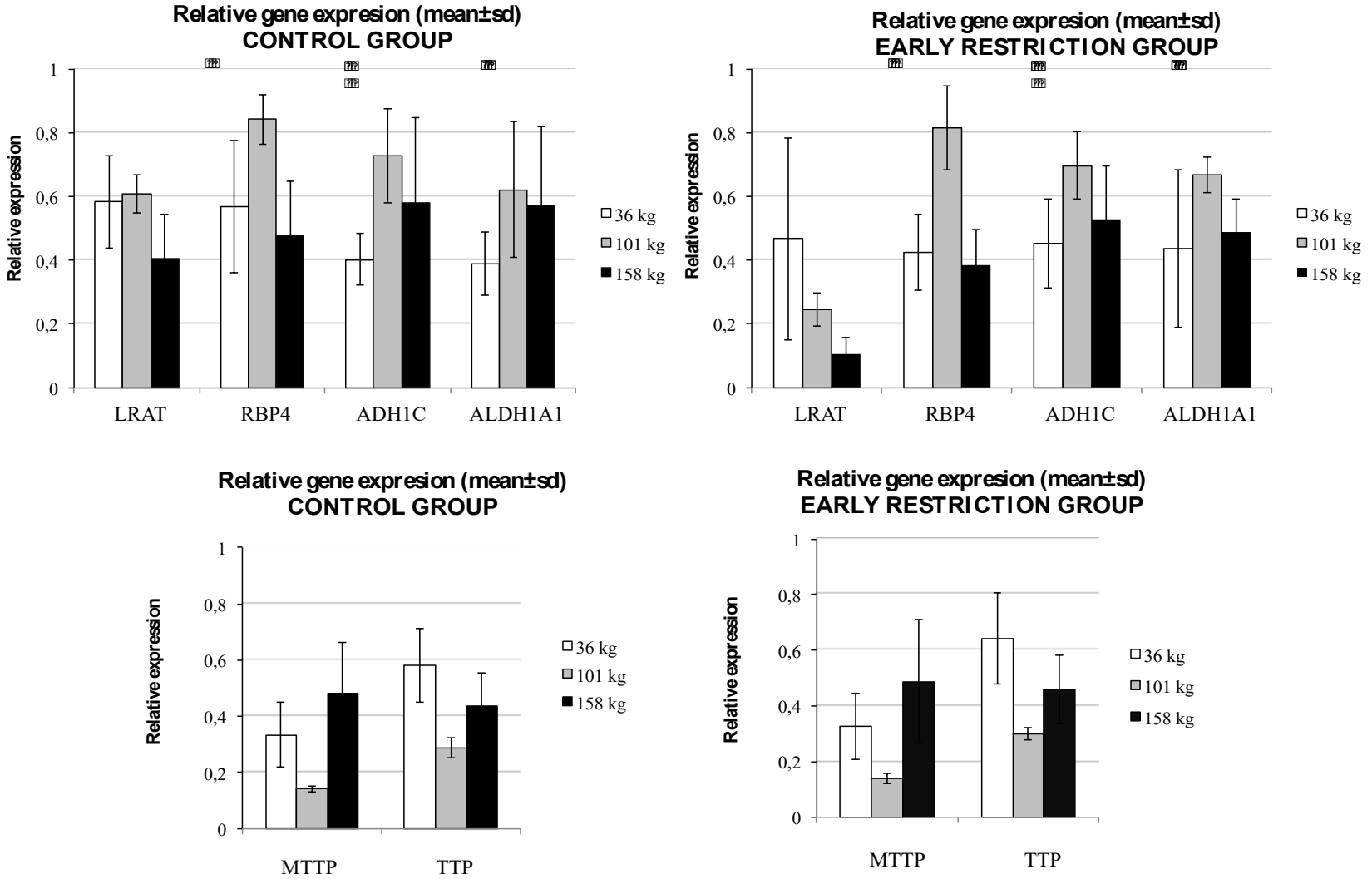


Expression of retinol and α-tocopherol metabolism-related genes as affected by the stage of growth

Relative expression of the previously mentioned genes at three different growing stages (36 kg, 101 kg and 158 kg LW) was compared in both CONTROL and ER groups (Figure 4). CONTROL and ER

groups showed similar relative expression pattern for *RBP4*, *ADH1C* and *ALDH1A1* depending on pig slaughter weight significantly higher when compared to their relative expression at 36 kg LW (P=0.0201, P=0.0014 and P=0.0157, respectively in CONTROL group and P=0.0009, P=0.0086 and P=0.0074, respectively in ER group). Moreover, the relative expression of *RBP4* and *ADH1C* decreased at 158 kg LW to reach similar levels to that found at 36 kg LW (P=0.244 and P=0.1414, respectively in CONTROL group and P=0.3022 and P=0.5437, respectively in ER group); nonetheless, *ALDH1C* expression did not change significantly from 101 to 158 kg LW, as a result, the expression level at this age was intermediate and not significantly different from the previous two growing stages in both CONTROL and ER groups. Concerning *LRAT* gene expression, the CONTROL group showed no difference at 36 kg LW compared to 101 kg LW. However, it is of interest to observe that in the ER group a trend to a decrease in *LRAT* expression between 36 kg LW and 101 kg LW was detected (P=0.0527). Moreover, the expression of *LRAT* decreased as observed in the other studied genes at the end of the fattening phase compared to 101 kg LW in both groups (P=0.0458 in CONTROL and P<0.0001 in ER group).

Figure 4- Relative expression of *ADH1C, ALDH1A1, LRAT , RBP4, MTTP* and *TTP* genes in liver from pigs fed a vitamin A-enriched diet (10,000 IU/kg diet) (CONTROL) or vitamin A-restricted diet (0 IU/kg diet) (ER) at different growing stages (36, 101 and 158 kg).



Concerning α-tocopherol-related gene expression as affected by pig slaughter weight (Figure 4), CONTROL and ER groups showed similar relative expression patterns and it was opposite to that observed for the relative expression of vitamin A-related genes. Hence, *MTTP* had the lowest expression levels at 101 kg LW, which was significantly lower when compared to their relative expression at 36 kg LW and 158 kg LW ($P<0.0001$and $P<0.0001$, respectively in the CONTROL group and $P<0.0001$ and $P<0.0001$, respectively in the ER group). A similar pattern was observed for *TTP* gene expression, ($P<0.0001$ and $P=0.0110$, respectively in the CONTROL group and $P<0.0001$ and $P=0.0002$, respectively in the ER group).

3.3.5- Discussion

Vitamin A is well known as an important regulatory factor involved in growth and development (Blaner and Olson, 1994). In the present work, pigs fed vitamin A-restricted diets (ER and LR) had lower ADG and worse feed efficiency than those given a vitamin A-enriched diet (CONTROL) during the growing period. It has been previously described in pigs that vitamin A deficiency may cause reduced weight gain and body vitamin A storage (NRC, 2012). Vitamin A deficiency has also been related to lower growth in children (West et al., 1997). However, other authors did not find any effect of vitamin A withdrawal on performance parameters of pigs (Chin et al., 2002; D'Souza et al., 2003; Olivares et al., 2009a; Olivares et al., 2009b; Olivares et al., 2011), mice (Sagazio et al., 2007) or ruminants (Arnett et al., 2007; Gorocica-Buenfil et al., 2008; Kruk et al., 2008; Pickworth et al., 2012). It has to be taken into account that in the present study piglets started dietary treatment at 16.3 kg LW, while other authors conducted their experiment in 23.7 kg LW (D'Souza et al. 2003) or 60 kg LW pigs (Olivares et al., 2009b).

In the present experiment, CONTROL pigs were fed a 7.5xNRC vitamin A-supplemented diet, which allowed pigs to store vitamin A both as retinol and as retinyl palmitate in body tissues. According to Shöne (1986) hepatic retinol concentrations > 30 IU /g indicate optimal supply for growth performance. These concentrations were reached in the CONTROL group of our study at the end of the growing period at 101 kg LW. Retinol was mainly accumulated in liver and, to a much lower degree, in adipose tissue. At 36 kg LW, after eight weeks of dietary vitamin A supplementation, retinol depots were 20-fold higher in liver than fat. These results are in agreement with those of Olivares et al. (2011). Moreover in the current study, this difference increased up to 70-fold after nine months of dietary treatment. However, retinol storage represents a small proportion of total retinoid depots in the body. Retinyl palmitate is the main form for vitamin A accumulation and is found mainly in the liver; as much as 90–95% of hepatic retinoid is stored as retinyl ester in lipid droplets of hepatic stellate cells in adult mice (Blaner and Olson, 1994). In agreement with this finding, we found 12-fold greater accumulation of retinyl palmitate than retinol in liver. Schweigert

et al. (2001) and Olivares et al. (2009b) feeding pigs with 12,300 IU vitamin A/kg and 13,000 IU vitamin A/kg, respectively, found retinyl palmitate concentrations around 14-17 times higher than retinol. However, most of the studies focused on vitamin A accumulation have been carried out using different genetic backgrounds, doses and during specific growing period, making comparisons between studies difficult. To our knowledge there are no previous studies on the evolution of retinol depots with age or growing time in pigs fed a 7.5xNRC vitamin A supplemented diet or on the possible effects of dietary vitamin A withdrawal at different periods. It is very interesting to observe in the present research that the retinol increase in hepatic and fat depots and retinyl palmitate accumulation in CONTROL animals were more marked during the growing phase than during fattening even though pigs had higher intake during this latter period. The rate of hepatic retinol accumulation was estimated to be at approximately 0.4 μg/g of retinol per kg LW during the growing period and 0.12 μg/g of retinol per kg LW during fattening. These results may indicate a saturation of retinol and retinyl palmitate depots with the supplementation time.

On the other hand there is little information about how vitamin A storage might be mobilized over time in order to maintain physiological levels of serum retinol in pigs. In our study, retinyl palmitate concentration was the most affected in restricted animals as observed in the negative slope of the regression equations (Table 4), followed by hepatic and fat retinol. These results are consistent with those previously reported by Olivares et al. (2009b) in pigs fed 13,000 IU vitamin A/kg for six weeks and then subjected to vitamin A depletion for five weeks prior to slaughter. Nevertheless, in that study the evolution of retinol depots over time was not assessed. In the present study, the retinol mobilization response was higher in fat and hepatic tissues than the accumulation response. At the end of the experiment, after nine months of dietary vitamin A supplementation, CONTROL pigs showed 1.4-fold and 5-fold increase in fat and hepatic retinol respectively. A decrease of 4-fold and 8-fold, respectively, was observed in ER. It is noticeable the high individual variability observed in liver retinol accumulation in supplemented pigs at the end of the fattening phase. This phenomenon has been previously reported in the literature (Schweigert et al., 2001; Siebert et al., 2006) and could be related to the high metabolic activity of the hepatic tissue that result in the accumulation of vitamin A as different compounds (retinol, retinyl palmitate and other esters). Hence, retinyl palmitate in the CONTROL group increased 7-fold while it decreased 8-fold in the ER group during the same period, which suggests a greater mobilization than accumulation response in fat and hepatic retinol compared to hepatic retinyl palmitate. Olivares et al. (2009b) also observed a faster mobilization of hepatic retinol (7-fold) than hepatic retinyl palmitate (4-fold) after vitamin A withdrawal of five weeks. When the effect of vitamin A restriction timing was compared (ER vs. LR), negative slopes of the regression equations in retinol fat depots were similar (Table 4) and not statistically different. For liver retinyl palmitate mobilization, both the slope and the average concentrations at 101 and 158 kg LW were similar for

the ER and LR groups. However, retinol depletion in liver was almost three times greater ($P<0.05$) in the LR group than in ER group, with both groups reaching similar retinol concentrations at 158 kg LW. Again, the slope for liver retinol depletion was numerically greater than for adipose tissue depletion in ER and LR groups. These results would indicate a different sensitivity between mobilization of vitamin A depots to maintain retinol homeostasis, suggesting a faster retinol mobilization from the liver. This phenomenon has been previously mentioned in the literature for beef cattle (Pickworth et al., 2012). Those authors found in steers a trend to lower fat and hepatic retinol concentration in those animals fed the non-vitamin A-supplemented diet for the whole experimental period; however in steers fed the restricted diet for the last 115 days, retinol was mainly mobilized from liver deposits.

Removing vitamin A from diet also resulted in higher α-tocopherol in fat and liver (Figure 2). An α-tocopherol increase in tissues might have a positive effect on meat quality (Dirinck et al., 1996). Olivares et al. (2009b) found higher concentrations of α-tocopherol when vitamin A was removed from the vitamin mix five weeks prior to slaughter in pigs fed diets with 13,000 IU vitamin A/kg from 60 kg to 100 kg LW. Ching et al. (2002) also reported a decrease in vitamin E levels by 13,200 IU/kg of dietary vitamin A supplementation in weaning pigs when compared to a group fed 2,200 IU/kg. However, Hoppe et al. (1992) and Anderson et al. (1995) did not find any interaction when diets were supplemented with 10,000 IU/kg or 20,000 IU/kg vitamin A in growing-finishing pigs, respectively. It has been suggested that dietary vitamin A modulates the concentration of α-tocopherol in plasma lipoproteins (Ametaj et al., 2000). However other authors reported an absorption interaction between both vitamins (Bieri et al., 1981). In the present study it is of interest to note that in liver, the ER group had a higher α-tocopherol increase than the LR group (14-fold vs. 7-fold slope increase) compared to CONTROL. According to the antagonism between vitamin A and α-tocopherol reported previously in the literature (Chin et al., 2002; Olivares et al., 2009b), this result would be expected since vitamin A deposition was lower in the ER than in the LR group. Conversely, α-tocopherol did not increase similarly in all tissues after vitamin A withdrawal. In fat, the increase in α-tocopherol levels was more marked in the LR than in the ER group ($P<0.0001$). Hence, providing a vitamin A-restricted diet to heavy pigs for 9 months (from 2 to 11 months of age) would be less effective for increasing fat α-tocopherol concentration than when the same diet is given for 7 months (from 4 to 11 months of age). Moreover, the LR group showed a 7-fold slope increase in α-tocopherol in fat when compared to the CONTROL group, similarly as reported for liver, (Table 4). This is the first study in which the effect of a vitamin A-restricted diet on tissue α-tocopherol deposition is evaluated and the results reported here might indicate that not only treatment duration but also tissue and timing of dietary vitamin A supplementation could be responsible for the effects on α-tocopherol accumulation.

Vitamin A activation, transport and accumulation are highly regulated due to its important role in different physiological processes (Wolf, 2001). We assessed the expression of four genes involved in vitamin A metabolism in liver, which plays a central role in vitamin A physiology (Frey and Vogel, 2011) and transport. Hepatic *ADH1C* and *LRAT* genes expression was significantly higher in CONTROL pigs than in the ER group at 101 kg LW. *ADH1C* and *ALDH1A1* were also numerically higher, although no statistical differences were found at 158 kg LW (Figure 3). There is no information on how dietary vitamin A affects *ADH1C* expression in mammals. *ADH1C* and *ALDH1A1* code for two enzymes that catalyze the synthesis of retinoic acid, the active metabolite of vitamin A. First, retinol is reversibly oxidized to retinaldehyde (ADHs enzyme family) and second, retinaldehyde is irreversibly oxidized to retinoic acid (RALDH enzyme family) (Molotkov and Duester, 2003). Moreover, *ADH1C* is found not only in liver but also in several retinol target tissues, which indicates its role in retinoid signaling (Duester, 2000). Consequently, the results observed are consistent with the role in maintaining retinol and retinoic acid steady-state levels in order to avoid both deficiencies in retinoic acid and excess in retinol, which may cause toxicity, especially in adult animals (Molotkow and Duster, 2003). When retinol concentrations exceed physiological levels, not only is the oxidation pathway activated, but also the esterification pathway controlled by *LRAT* becomes activated. This enzyme is present in microsomal fractions of several tissues that contain high levels of retinyl esters. Consistent with the results found for the oxidation enzymes, *LRAT* gene expression was upregulated in CONTROL animals at 101 and 158 kg LW. Dietary vitamin A and retinoic acid have been proposed to regulate vitamin A metabolism-related enzymes, specifically *LRAT* (Wolf, 2001). In the ER group, due to the lack of vitamin A intake, *LRAT* expression would be inhibited, leading to big differences between CONTROL and ER groups, with 2.3- and 3.6-fold changes in expression levels at 101 and 158 kg LW, respectively. These results are in agreement with those previously observed in lung of adult rats, in which *LRAT* mRNA levels in vitamin A restricted animals were <10% of those found in vitamin A-supplemented animals (Zolfaghari and Ross, 2002). Moreover, in the present experiment it is also worth noting that only *LRAT* expression was directly related to dietary vitamin A supplementation. Ross and Zolfaghari (2004) reported previously that in long-term feeding studies *LRAT* is expressed in a dose-dependent way across a wide range of dietary vitamin A intakes.

The last gene assessed related to vitamin A is *RBP4*. It is abundantly synthesized by hepatocytes and to a lesser degree by adipose cells (Blaner, 1989; Tsutsumi et al., 1992) and then secreted only in the retinol-bound form when retinol is released from the liver into the bloodstream to meet the retinoid needs of other tissues (Goodman, 1984a). There was no difference in *RBP4* gene expression due to dietary treatment, which is consistent with a maintained retinol homeostasis, by releasing stored retinol into bloodstream at the expense of retinol and retinyl esters depots in liver and fat (Pickworth et al., 2012). It has been reported that blood levels of retinol-RBP in both

humans and animals are maintained very constant, except in extreme cases and in certain disease states (Goodman, 1984b; Biesalski et al., 1999).

The relative expression of *ADH1C, ALDH1A1, LRAT* and *RBP4* were also studied as affected by timing (Figure 4). This is the first study in which the expression of *ADH1C, ALDH1A1* and *RBP4* were measured at different pig ages or growing times. These results indicate a higher activity of these genes at 101 kg LW, which is in accordance with the higher accumulation of vitamin A during the growth period that could lead to the observed differences in performance parameters. These results are very interesting and indicate that timing of dietary treatment should be taken into consideration. Moreover, the expression of *LRAT* as affected by growing time was higher at 36 and 101 kg LW when compared to 158 kg-pigs in both CONTROL and ER groups. Ross and Zolfaghari (2004) found a lack of significant increase over time in *LRAT* mRNA in rats fed a vitamin A-supplemented diet (50 mg retinol/kg feed) and concluded that the total *LRAT* enzyme activity in normal rat liver is sufficient to esterify all of the retinol entering the liver from the absorption of a standard (4 mg retinol/ kg feed) or moderately (12-fold the standard diet) vitamin A-supplemented diet.

The expression of *MTTP* and *TTP* genes related to α-tocopherol metabolism, no clear effect of dietary vitamin A treatment was found. The relative expressions of *MTTP* and *TTP* genes were also affected by the timing, but showed an opposite pattern to that observed for vitamin A-related genes. The expression of vitamin A related genes increased at 101 kg LW, whilst a decrease was observed in the α-*TTP,* which plays an important role in the circulation of plasma α-tocopherol (Zuo et al., 2014) and *MTTP*, which participates in the secretion of vitamin E via chylomicrons (Anwar et al., 2007). The mechanism by which vitamin A and E interact is still unknown. The results of the present study suggest that the interaction between both vitamins is not directly dependent on dietary vitamin A supplementation but on the growth timing, probably in relation to a specific body composition or physiological maturation. It has been reported that secretion of vitamin E via chylomicrons was dependent on the availability of oleic acid and *MTTP* activity (Anwar et al., 2007). These authors also found that lipids induced the secretion of vitamin E with intermediate density lipoproteins. On the other hand, Kiefer et al. (2012) reported that *ALDH*1 regulates fat in rodents and Olivares et al. (2011) found that withdrawal of dietary vitamin A not only increased vitamin E deposition but also oleic acid and other monounsaturated fatty acids. Consequently, regulation of both vitamins in the organism might be related to fat or fatty acid deposits or with their effects on lipid metabolism.

3.3.6- Conclusions

In conclusion, the expression of some genes involved in retinoic acid (*ADH1C*) and retinyl palmitate formation (*LRA*T) was higher in pigs receiving a 7.5x NRC dietary vitamin A enrichment after six months of supplementation (101 kg LW) when compared to those fed un-supplemented diets. However, only *LRAT* was expressed in a dose-dependent way by long-term vitamin A supplementation. Retinol and thus α-tocopherol depots seemed to be more sensitive to dietary vitamin A modifications during the growing period around 101 kg LW that could be related to differences in the expression of *RBP*4, *ADH1C, ALDH1A1, MTTP* and *TTP* at 101 kg LW. Our results suggest that the use of 7.5xNRC dietary vitamin A supplementation for long periods is not needed and short or long-term vitamin A withdrawal has the potential to reduce feed costs and increase fat and liver α-tocopherol levels without adverse effect on overall growth performance in heavy pigs.

Acknowledgements

The authors are grateful to the CIA Deheson del Encinar (Toledo). De la Torre, I. technical support is also acknowledged. This research was supported by Comisión Interministerial de Ciencia y Tecnología (AGL2010-21991).

3.4 CAPITULO 4: La restricción de vitamin A en la dieta modifica la diferenciación de adipocitos y la composición de ácidos grasos de la grasa intramuscular en cerdos ibéricos

---

Dietary vitamin A restriction affects adipocyte differentiation and fatty acid composition of intramuscular fat in Iberian Pigs

Ayuso M, Óvilo C, Rodríguez-BertosA, Rey AI, Daza A, Fenández A, González-Bulnes A, López-Bote CJ and Isabel B.

Meat Science. 2015;108:9-16.

Meat Science 108 (2015) 9–16

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

Dietary vitamin A restriction affects adipocyte differentiation and fatty acid composition of intramuscular fat in Iberian pigs

M. Ayuso [a,*], C. Óvilo [b], A. Rodríguez-Bertos [c,d], A.I. Rey [a], A. Daza [e], A. Fenández [b], A. González-Bulnes [f], C.J. López-Bote [a], B. Isabel [a]

[a] Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain
[b] Departamento de Mejora Genética Animal, INIA, 28040 Madrid, Spain
[c] Departamento de Medicina y Cirugía, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain
[d] Animal Health Surveillance Center (VISAVET), Complutense University of Madrid, 28040 Madrid, Spain
[e] Departamento de Producción Animal, ETSIA, Universidad Politécnica de Madrid, 28040 Madrid, Spain
[f] Departamento de Reproducción Animal, INIA, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 13 November 2014
Received in revised form 13 April 2015
Accepted 27 April 2015
Available online 5 May 2015

Keywords:
Fatty acid
Iberian pig
Intramuscular fat
Preadipocyte
Vitamin A

ABSTRACT

The aim of this study was to investigate whether dietary vitamin A level is associated with differences in adipocyte differentiation or lipid accumulation in Iberian pigs at early growing (35.8 kg live weight) and at finishing (158 kg live weight). Iberian pigs of 16.3 kg live weight were allocated to two feeding groups, one group received 10,000 IU of vitamin A/kg diet (control); the other group received a diet with 0 IU of vitamin A (var) for the whole experimental period. The dietary vitamin A level had no effect on growth performance and carcass traits. The early suppression of vitamin A increased the preadipocyte number in *Longissimus thoracis* (LT) muscle in the early growth period ($P < 0.001$) and the neutral lipid content and composition (higher MUFA and lower SFA content) at the end of the finishing period ($P < 0.05$). Vitamin A restriction in young pigs increases their lipogenic potential without affecting carcass traits.

1. Introduction

Adipocyte differentiation is an important factor for fat accumulation in the body. Adipocytes are derived from fibroblast-like preadipocytes and grow in size by accumulation of lipids in the cytoplasm in association with terminal differentiation (Hausman, Campion, & Martin, 1980). In the early stage of adipocyte differentiation, many adipocyte characteristic genes are sequentially activated and play established roles in promoting the differentiation process (Ntambi & Kim, 2000).

Adipocyte differentiation is regulated by various kinds of hormones (Boone, Gregoire, & Remacle, 2000; Gregoire, Smas, & Sul, 1998). Furthermore, it is well known that fat-soluble vitamins, especially metabolites of vitamin A and D, modulate adipocyte differentiation in cultured cells in mammals (Kawada et al., 1990). All-*trans* retinoic acid (RA, the active metabolite of vitamin A) and 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) inhibit adipocyte differentiation in cultured cells at a supraphysiological concentration (Kawada et al., 1990; Sato & Hiragun, 1988; Suryawan & Hu, 1997). However, very low concentration (1 pM − 10 nM) of RA stimulates adipocyte differentiation (Safonova et al., 1994).

Due to its role in reproduction, growth, development and immune response, commercial pig diets in the European Union contain vitamin A concentrations approximately six- to ten-fold higher than NRC recommendation (1317 IU/kg diet) (Fraga and Villamide, 2000). However, studies in vivo showed that a dietary level of 1300 IU of vitamin A for 11 weeks was associated with a higher intramuscular fat (IMF) content in *Longissimus thoracis* (LT) muscle than a diet with 13,000 IU in pigs (Olivares, Reya, Daza, & Lopez-Bote, 2011). However, in another experiment, Olivares, Daza, Rey, and Lopez-Bote (2009a) found no effect of dietary vitamin A on IMF content in pigs. Thus, the effect of vitamin A on body fat accumulation in swine remains unclear. Also, previous studies have found that dietary vitamin A concentration alters fatty acid composition of adipose tissue in sheep (Daniel, Salter, & Buttery, 2004), beef (Siebert et al., 2006) and pigs (Olivares, Daza, Rey, & Lopez-Bote, 2009b; Olivares et al., 2009a, 2011) but no effect was found on the fatty acid composition of IMF in pigs (Olivares et al., 2011). These experiments have been performed with different animals (ruminant vs. non-ruminant), genotypes (Duroc vs. lean pigs) and different times of supplementation or restriction of vitamin A. Both, IMF content and fatty acid composition are determinant factors affecting meat quality (Wood et al., 2008) and they are of special interest in high quality meat products, such as those obtained from Iberian pigs. Moreover, the effects of dietary vitamin A level have never been

* Corresponding author. Tel.: +34 913943781.
E-mail address: mayuso@ucm.es (M. Ayuso).

http://dx.doi.org/10.1016/j.meatsci.2015.04.017
0309-1740/

3.4.1- Abstract

The aim of this study was to investigate whether dietary vitamin A level is associated with differences in adipocyte differentiation or lipid accumulation in Iberian pigs at early growing (35.8 kg live weight) and at finishing (158 kg live weight). Iberian pigs of 16.3 kg live weight were allocated to two feeding groups, one group received 10,000 IU of vitamin A/kg diet (CONTROL); the other group received a diet with 0 IU of vitamin A (VAR) for the whole experimental period. The dietary vitamin A level had no effect on growth performance and carcass traits. The early suppression of vitamin A increased the preadipocyte number in *Longissimus thoracis* (LT) muscle in the early growth period ($P < 0.001$) and the neutral lipids content and composition (higher MUFA and lower SFA content) at the end of the finishing period ($P < 0.05$). Vitamin A restriction in young pigs increases their lipogenic potential without affecting carcass traits.

Keywords: Fatty Acid, Iberian Pig, Intramuscular Fat, Preadipocyte, Vitamin A.

3.4.2- Introduction

Adipocyte differentiation is an important factor for fat accumulation in the body. Adipocytes are derived from fibroblast-like preadipocytes and grow in size by accumulation of lipids in the cytoplasm in association with terminal differentiation (Hausman, Campion, & Martin, 1980). In the early stage of adipocyte differentiation, many adipocyte characteristic genes are sequentially activated and play established roles in promoting the differentiation process (Ntambi & Kim, 2000). Adipocyte differentiation is regulated by various kinds of hormones (Boone, Gregoire, & Remacle, 2000; Gregoire, Smas, & Sul, 1998). Furthermore, it is well known that fat-soluble vitamins, especially metabolites of vitamin A and D, modulate adipocyte differentiation in cultured cells in mammals (Kawada et al., 1990). All-*trans* retinoic acid (RA, the active metabolite of vitamin A) and 1,25-dihydroxyvitamin D3 (1,25 (OH)2D3) inhibit adipocyte differentiation in cultured cells at a supraphysiological concentration (Kawada et al., 1990; Sato & Hiragun, 1988; Suryawan & Hu, 1997). However, very low concentration ($1pM^{-10}nM$) of RA stimulates adipocyte differentiation (Safonova et al., 1994).

Due to its role in reproduction, growth, development and immune response, commercial pig diets in the European Union contain vitamin A concentrations approximately six- to ten-fold higher than NRC recommendation (1,317 IU/kg diet) (Fraga and Villamide, 2000). However, studies in vivo showed that a dietary level of 1,300 IU of Vitamin A for 11 weeks was associated with a higher intramuscular fat (IMF) content in *Longissimus thoracis* (LT) muscle than a diet with 13,000 IU in pigs (Olivares, Reya, Daza, & Lopez-Bote, 2011). However, in another experiment, Olivares, Daza, Rey, and Lopez-Bote (2009a) found no effect of dietary vitamin A on IMF content in pigs. Thus, the effect of Vitamin A on body fat accumulation in swine remains unclear. Also, previous studies have found that dietary vitamin A concentration alters fatty acid composition of adipose tissue in sheep (Daniel, Salter, & Buttery, 2004), beef (Siebert et al., 2006) and pigs (Olivares et al., 2009a; Olivares, Daza, Rey, & Lopez-Bote, 2009b; Olivares et al., 2011) but no effect was found on the fatty acid composition of IMF in pigs (Olivares et al., 2011). These experiments have been performed with different animals (ruminant vs. non-ruminant), genotypes (Duroc vs. lean pigs) and different times of supplementation or restriction of vitamin A. Both, IMF content and fatty acid composition are determinant factors affecting meat quality (Wood et al., 2008) and they are of special interest in high quality meat products, such as those obtained from Iberian pigs. Moreover, the effects of dietary vitamin A level have never been assessed in this breed. On the other hand, Iberian pigs have a high adipogenic and lipogenic potential, which could modify the effects of vitamin A restriction on fatness and thus, the use of dietary vitamin A restriction as a strategy to increase IMF should be tested in Iberian pigs.

Several authors have established that vitamin A exerts its effects on adipose tissue via regulation of expression of genes involved in adipogenesis and lipid metabolism (Bonet, Ribot, Felipe, & Palou, 2003; Daniel et al., 2004; Fernandez et al., 2011; Schwarz, Reginato, Shao, Krakow, & Lazar, 1997). Indeed, vitamin A, and specifically its metabolite, RA, is well known as a potent transcriptional regulator. Balmer and Blomhoff (2002) established that more than 500 genes are regulated by RA. Many genes are involved in the adipogenic process, from the very beginning, when *C/EBPs* and *PPARs* (mainly *PPARG*) families are induced, to the final differentiation process, when mature adipocytes express genes involved in lipid metabolism such as ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase, fatty acid synthase and others (Gregoire et al., 1998; Rosen, Walkey, Puigserver, & Spiegelman, 2000). The expression of these genes is considered a signal of the mature adipocyte phenotype. However, most of the available data in the literature is obtained from adipocyte culture studies. Results coming from studies *in vivo* are scarce and to our knowledge, there is no information about the effect of a restriction of dietary vitamin A on gene expression in pigs.

The objective of this study was to investigate how dietary vitamin A restriction affects gene expression in young pigs (35.8 kg) and impacts adipocyte differentiation and lipid accumulation in Iberian pigs at early growth (35.8 kg) and finishing (158 kg) periods.

3.4.3- Materials and methods

Animals and diets

Animal manipulations were done in compliance with the regulations of the Spanish policy for animal protection RD1201/05, which meets the European Union directive 86/609 about the protection of animals used in experimentation. The experiment was specifically assessed and approved (report CEEA 2010/003) by the Spanish National Institute for Agricultural and Food Research and Technology (INIA) Committee of Ethics in Animal Research. The trial was conducted at CIA Dehesón del Encinar (Oropesa, Toledo, Spain).

Thirty-eight castrated male (Torbiscal Pure Iberian) were randomly selected from a population. They were weaned at four weeks of age at a live weight (LW) of 11.7 ± 2.2 kg and were housed in pens until 2 months of age (average weight of 16.3 ± 2.5 kg) when piglets were randomly assigned to the two treatment groups, housed individually and given the experimental diets. One group was fed a vitamin A-enriched starter diet (10,000 IU vitamin A/kg diet) (CONTROL) and the other group received a starter diet formulated with no vitamin A (VAR, the same content in all periods) added in the premix (Table 1) from 16.3 ± 2.5 kg LW to 32.2 ± 4.5 kg LW. Diets were adjusted to meet requirements depending on the growing period. Pigs were changed to the corresponding control (10,000 IU vitamin A/kg diet) and vitamin A-restricted growing (from 32.2 ± 4.5 kg LW to 101 ± 4.1 kg LW) and finishing diets (from 101 ± 4.1 kg LW to 158 ± 7 kg LW). Pigs were fed 3.5 % LW restriction until four months of age, 3% LW restriction until eight months and 2.5% LW restriction from this age until slaughter. Pigs had *ad libitum* access to water.

Ingredients, chemical composition and main fatty acids of experimental diets are shown in Table 1. Diets were formulated according to general guidelines proposed by De Blas, Gasa, and Mateos (2013) for Iberian pigs.

Table 1. Ingredient composition, calculated analysis (g/kg, as-fed basis unless stated otherwise) and fatty acid composition of the experimental diets.

	Starter		Growth		Finishing	
	CONTROL[a]	VAR[b]	CONTROL	VAR	CONTRO	VAR
Ingredient						
Barley	280.0	280.0	500.0	500.0	453.2	453.2
Soybean meal	155.1	155.1	169.4	169.4	75.9	75.9
Wheat	250.0	250.0	290.3	290.3	300.0	300.0
Soybean protein	25.0	25.0				
Corn	194.9	194.9				
Whey powder,	25.0	25.0				
Full fat soybean	20.0	20.0				
High oleic					120.0	120.0
Lard	17.0	17.0	10.0	10.0	20.0	20.0
Calcium carbonate	5.4	5.4	8.2	8.2	8.2	8.2
Dicalcium	13.6	13.6	12.0	12.0	12.0	12.0
CONTROL-Mineral	4.0	0	4.0	0	4.0	0
VAR-Mineral and	0	4.0	0	4.0	0	4.0
Salt	4.0	4.0	4.5	4.5	4.0	4.0
L-Lysine (500 g/kg)	4.0	4.0	1.6	1.6	2.2	2.2
Methionine-OH	1.4	1.4				
L-Threonine	0.6	0.6				
Calculated analysis[e]						
Net energy (MJ/kg)	10.0	10.0	9.5	9.5	10.4	10.4
Crude protein	178.2	178.2	171.9	171.9	147.0	147.0
Crude fat	41.9	41.9	29.0	29.0	85.0	85.0
Crude fiber	35.7	35.7	40.9	40.9	55.2	55.2
Crude Ash	48.0	48.0	49.2	49.2	49.2	49.2
Fatty acid composition (g/100 g total fatty acids)						
C12:0	1.8	1.6	7.2	8.5	1.0	1.0
C14:0	2.0	2.0	3.6	3.5	1.1	1.2
C16:0	18.8	19.2	18.0	17.8	11.2	11.8
C16:1 n-9	0.1	0.8	0.8	0.9	0.6	0.7
C16:1 n-7	1.0	0.4	0.3	0.3	0.2	0.2
C17:0	0.5	0.2	0.0	0.0	0.1	0.1
C17:1	0.2	0.6	0.0	0.0	0.2	0.2
C18:0	6.4	5.8	3.9	4.2	3.9	4.2
C18:1 n-9	26.6	26.3	21.1	20.5	57.0	57.5
C18:1 n-7	1.7	1.4	1.3	1.3	0.2	0.2
C18:2 n-6	36.2	36.8	39.0	37.8	21.4	20.1
C18:3 n-3	3.1	3.3	3.6	3.9	1.5	1.5
C20:0	0.3	0.3	0.3	0.3	0.4	0.3
C20:1 n-9	0.6	0.6	0.6	0.6	0.6	0.6
C20:3 n-6	0.2	0.2	0.2	0.2	0.3	0.2
C20:4 n-6	0.5	0.4	0.2	0.2	0.4	0.2
SFA[f]	29.8	29.1	32.9	34.3	17.6	18.7
MUFA[g]	30.2	30.1	24.1	23.6	58.8	59.4
PUFA[h]	40.0	40.7	43.0	42.1	23.6	22.0

[a]CONTROL = CONTROL diet. 10,000 IU vitamin A/kg feed supplementation.
[b]VAR= Vitamin A restricted diet. 0 IU vitamin A/kg feed supplementation.
[c]CONTROL-Mineral and vitamin premix provided per kg of feed: Vitamin A, 10,000 IU; Vitamin D3, 2,000 IU; Vitamin E, 26.7 mg; Vitamin B1, 1.3 mg; Vitamin B2, 4.0 mg; vitamin B12, 0.020 mg; Vitamin B6, 1.3 mg; Calcium pantothenate, 13.3 mg; Nicotinic acid, 20 mg; Biotin, 0.1 mg; Folic acid, 0.1 mg; Vitamin K3, 2 mg; Fe, 133.3 mg; Cu, 26.7 mg; Co, 0.30 mg; Zn, 133.3 mg; Mn, 76.7 mg; I, 1.3 mg; Se, 0.30 mg; Ethoxyquin, 150 mg.
[d]VAR-Mineral and vitamin premix provided per kg of feed: Vitamin A, 0 IU; Vitamin D3, 2,000 IU; Vitamin E, 26.7 mg; Vitamin B1, 1.3 mg; Vitamin B2, 4.0 mg; vitamin B12, 0.020 mg; Vitamin B6, 1.3 mg; Calcium pantothenate, 13.3 mg; Nicotinic acid, 20 mg; Biotin, 0.1 mg; Folic acid, 0.1 mg; Vitamin K3, 2 mg; Fe, 133.3 mg; Cu, 26.7 mg; Co, 0.30 mg; Zn, 133.3 mg; Mn, 76.7 mg; I, 1.3 mg; Se, 0.30 mg; Ethoxyquin, 150 mg.
[e]According to Fundación Española DesarrolloNutrición Animal (2010) (supplied per kg of diet).
[f]SFA (C12:0+C14:0+C16:0+C17:0+C18:0+C20:0); sum of saturated fatty acids.
[g]MUFA (C16:1n-9+C16:1n-7+C17:1+C18:1n-9+C18:1n-7+C20:1n-9); sum of monounsaturated fatty acids.
[h]PUFA (C18:2n-6+C18:3n-3+C20:3n-9+C20:4n-6); sum of polyunsaturated fatty acids.

Sample collection

Nine pigs per treatment were slaughtered at 4 months of age (early growing) and the remaining (n = 10) at 11 months of age (finishing) (Industrias Cárnicas Alonso, S.L., Toledo, Spain) when pigs reached the averaged weights of 35.8 ± 3.1 and 158 ± 7 kg LW, respectively. In the slaughterhouse, carcass length from the posterior edge of the *Symphysis pubica* to the anterior edge of the first rib, ham length from the anterior edge of the *Symphysis pubica* to the *articulatio tarsi*, and ham circumference at its widest point were measured on the left side of each carcass were determined with a tape measure. Backfat thickness at the 10th rib on the midline of the carcass (skin included) was also measured with a ruler. Samples from loin (LT) at the level of the last rib were taken, weighed, vacuum-packed in low-oxygen permeable film and kept frozen at –20º C until fatty acid composition analysis. Prior to fatty acid analysis, muscle samples were freeze dried for two days in a lyophilizer (Lyoquest, Telstar, Tarrasa, Spain) and grounded in a Mixer Mill MM400 (Retsch technology, Haan, Germany) until muscle was completely powdered. Loin samples for gene expression study were immediately frozen in liquid nitrogen and kept at -80°C until analysis.

Laboratory analysis

Fatty acid composition of diets

Fatty acids of diets were extracted and quantified by the one-step procedure of Sukhija and Palmquist (1988) from lyophilized samples. Fatty acid methyl esters were analyzed by a gas chromatograph (Hewlett Packard HP-6890, Avondale, PA, USA) with a flame ionization detector and a capillary column (HP-Innowax, Agilent Technologies Gmbh, Germany) as previously described (Lopez-Bote, Rey, Isabel, & Sanz, 1997). A temperature program of 170 to 245 ºC was used. The injector and detector were maintained at 250 ºC. The carrier gas (helium) flow rate was 2 mL/min. Results were expressed as gram per 100 grams of detected FAMEs.

Neutral and polar lipids content and fatty acid composition of samples

Longissimus thoracis muscle lipids were extracted as proposed by Segura and Lopez-Bote (2014) and separated into neutral lipids (NL) and polar lipids (PL) (main fractions of IMF) using aminopropyl minicolumns, following the method used by Ruiz, Antequera, Andres, Petron, and Muriel (2004). Lipid fractions extracts were methylated in the presence of sulphuric acid and analyzed by gas chromatography as described by Lopez-Bote et al. (1997).

The activity of stearoyl-CoA desaturase (SCD, 1.14.19.1) enzyme was estimated as C18:1/C18:0 and MUFA/SFA ratios (Hulver et al., 2005).

Tissue handling for immunohistochemistry

The muscle tissue (LT) was removed from the carcass at the level of the last rib. The tissue was divided into 2 to 4 cm sections cut perpendicular to the long axis of the body. The sections were fixed in Bouin´s fluid (saturated picric acid, buffered formalin stabilized with methanol to pH = 7.0 and glacial acetic acid) for up to two days. Large specimens were fixed for up to three days. After the fixing period the samples were transferred to 70% alcohol. If the tissue was still yellow in the paraffin block, the hydrated sections were placed in alkaline solution to remove residual picric acid, and then rinsed with water before staining. Then the specimen was embedded in paraffin, cut at 4 μm and routinely stained with haematoxylin and eosin (Pathology Service, Veterinary Teaching Hospital of Complutense University, Madrid, Spain) for routine examination (García del Moral, 1993).

Immunohistochemistry

Preadipocyte factor-1, pref-1, is a transmembrane protein that is part of the family of epidermal growth factor-like repeat-containing proteins that are involved in cell fate determination. Pref-1, which is coded by an adipocyte differentiation inhibitor gene, is highly expressed in preadipocytes, but its expression is completely abolished during differentiation into a mature adipocyte (Gondret, Perruchot, Tacher, Berard, & Bee, 2011, Huff et al., 2005). Several authors have proposed the immunohistochemical localization of pref-1 or its expression to quantify the amount of preadipocytes in different tissues (Deiuliis, Li, Lyvers-Peffer, Moeller, & Lee, 2006; Gondret et al., 2011; Huff et al., 2005). Thus, immunohistochemistry was used to investigate preadipocyte presence, marked with delta-like homolog (DLK1) also known as preadipocyte factor 1 (DLF/Pref-1 Polyclonal Antibody - Proteintech, Manchester, United Kingdom) (Huff, Lozeman, Weselake, & Wegner, 2005).

Preadipocyte cell detection was investigated using the StreptAvidin-Biotin Complex method as described by (Carrasco et al., 2011).

Sections were deparaffinised, rehydrated and further placed in a steel pressure cooker containing 2 L of 10 mM sodium citrate buffer (pH 6.0) and heated for 3 min after the maximum pressure had started, as the antigen unmasking protocol. The slides were cooled at room temperature in the same buffer for 20 min and washed in distilled water and Tris–buffered saline (TBS) (0.1 M Tris

base, 0.9% NaCl, pH 7.4). After this procedure, samples were incubated for 15 min with 1% H_2O_2 to block endogenous peroxidase activity, followed by a 5-min wash in distilled water and another 5-min wash in TBS. Previously, to add the primary antibody, the sections were incubated for 30 min with normal serum block (normal goat serum code no. X0901, Dako Cytomation, Glostrup, Denmark) at 1:30 (v/v) dilution. Then, slides were incubated overnight at 4 ºC with the primary antibody (polyclonal rabbit anti - DLF/Pref-1) at dilution of 1:100 (v/v). The slides were incubated with goat anti-rabbit biotinylated secondary antibody (1:400; v/v), 30 min at room temperature; E 0353, DakoCytomation, Glostrup, Denmark). Next, all the slides were incubated with StreptAvidin–biotin–peroxidase complex (1:400; v/v), 30 min at room temperature; P50242, Zymed, San Francisco, CA). All washes (5 min, two times between each incubation step) and dilutions were made in TBS buffer. Immunoreactivity was observed with 3,3'-diaminobenzidine tetrachloride (D5050, Sigma Chemical Co., St. Louis, MO) and H_2O_2 (0.01%) in distilled water. After washing in tap water for 10 min, slides were counterstained for 3 min with Carrazzi's hematoxylin, washed in tap water, dehydrated, and mounted. Negative control slides were included within each batch of slides, which were prepared in all cases by omitting the primary antibody and incubating tissue sections with TBS.

For evaluation of cells, an optical light microscope (Olympus BX50, Hamburg, Germany) was used with 10x magnification. Positive DLF/Pref-1 stained sections were counted in five random fields of each slide. Five photographs (Olympus DP50, Hamburg, Germany) in different fields of the histological section stained with the polyclonal rabbit anti-DLF/Pref-1 antibody were taken and analyzed with a computer image analyser (software Viewfinder Lite® version 1.0). After obtaining the photographs, the preadipocytes were identified and counted. Cells expressing DLF/Pref-1 showed nuclear brown staining.

Quantitative gene expression

A quantitative gene expression analysis was performed in LT muscle from early growing animals in a panel of seven candidate genes (Table 2). We selected a panel of 7 candidate genes, which are involved in RA signalling and transcriptional control of adipogenesis mediated by RA (*RARA, RXRG* and *CRABPII*) adipocyte differentiation (*CEBP, PPARG* and SREBP*1C*) and fatty acids metabolism (*SCD*). Retinoic acid is considered an inhibitor of adipocyte differentiation by inhibiting the *CEBPB* signalling pathway and thus, blocking the expression of *PPARG* (Schwarz et al., 1997), which is considered the master regulator of adipogenesis.

RNA was extracted from 50-100 mg frozen muscle tissue samples using the Ribopure kit according to the manufacturer´s instructions (Ambion, Austin, TX). The RNA concentration was quantified using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). RNA quality was evaluated by an Agilent Bioanalyzer 2100 device (Agilent Technologies, Palo Alto, USA). The RNA Integrity Number values ranged from 7.7 to 8.4. First-strand cDNA was synthesized using 1 µg of

total RNA as template treated with Superscript II (Invitrogen, Life Technologies, Paisley, UK) and random hexamers in a total volume of 20 µl.

Primer pairs used for quantification were designed using Primer Select software (DNASTAR, Wisconsin, USA) from the available GENBANK and/or ENSEMBL pig sequences, covering different exons in order to assure the amplification of the cDNA. Sequence of primers and amplicon lengths are indicated in Table 2.

Table 2: Primer design for qPCR, gene details and PCR efficiencies (%) in Longissimus thoracis muscle (Eff).

Gene name	Gene symbol	GenBank Acc.	Forward primer sequence	Reverse primer sequence	Size	Eff
Adipocyte differentiation						
CCAAT/enhancer binding protein β	CEBPB	NM001199889	GTGGCGCCGGCAAAACTT	GAGGGGGCAGGAGGAGAGGCAGAG	203	96.8
Peroxisome proliferator-activated receptor G	PPARG	DQ437884	GGCGAGGGCGATCTTGACAG	GATGCGAATGGCCACCTCTTT	148	99.9
Sterol regulatory binding	SREBP-1c	AY307771	TTGCGCAAGGCCATCGACTACA	GTCTACCACCTCCGGCTTCACA	180	94.7
Retinoic acid signalling and transcriptional control of adipogenesis						
Retinoic acid receptor α	RARA	XM00313147	TCCGCCGAAGCATCCAGAAGAA	ACCTCCGGCGTCAGCGTGTAGC	217	92.9
Retinoid X receptor G	RXRG	NM00113021	GGGGTTGGCTCCATCTTTGA	ACCTGCCCGGCTGTTCTG	223	95.6
Cellular retinoic acid binding protein 2	CRABP2	NM001164509	GTACCACGGAGATCAACTTCAA	TGCCGTCATGGTCAGGA	200	91.8
Fatty acids metabolism						
Stearoyl-CoA desaturase	SCD	JN613287	TCCCGACGTGGCTTTTTCTTCT	CTTCACCCCAGCAATACCAG	205	88.9

Standard PCRs (polymerase chain reactions) on cDNA were carried out to verify amplicon sizes. Transcript quantification was performed using SYBR Green mix (Roche, Basel, Switzerland) with a LightCycler480 (Roche, Basel, Switzerland) in 384-well Reaction Plates (Roche, Basel, Switzerland). The qPCR (quantitative polymerase chain reaction) reactions were prepared in a total volume of 20 µl containing 2.5 µl of cDNA (1/20 dilution), 10 µl of SYBR Green mix and 0.15 µM of both forward and reverse primers. As negative controls, mixes without cDNA were used. Cycling conditions were 95 ºC for 10 min, followed by 45 cycles of 95 ºC (15 s) and 60 ºC (1 min) where the fluorescence was acquired. Finally, a dissociation curve to test PCR specificity was generated by one cycle at 95 ºC (15 s) followed by 60 ºC (20 s) and ramp up to 95 °C with acquired fluorescence during the ramp to 0.01°C/s. Data were analyzed with LyghtCycler480 SW1.5 software (Roche, Basel, Switzerland). All samples were run in triplicate as technical replicates and dissociation curves were carried out for each individual replicate. Single peaks in the dissociation curves confirmed the specific amplification of the genes. PCR efficiency was estimated by standard curve calculation using four concentrations of cDNA five-fold dilutions from a pool of samples and calculated from: $E = 10^{-1/slope}$ .Values of PCR efficiency are indicated in Table 2. Average crossing points (C_p) values were employed for the statistical analyses of differential expression. Four commonly used housekeeping genes (*ACTB, B2M, GAPDH* and *TBP*) were tested with geNorm software (Vandesompele et al., 2002) to evaluate their stability based on the "M" value. *GAPDH* and *ACTB* were selected.

Statistical analysis

Phenotype data was analyzed as a completely randomized design using the general linear model (GLM) procedure contained in the SAS version 9.2 (2010). Dietary treatment was considered as systematic effect, and residual effects as random. The animal was the experimental unit for all data analysis. Live weight and performance data are presented as means ± SD. For statistical analysis of performance parameters, initial weight was used as covariate when it was significant and removed from the model when not.

Statistical analysis of gene expression data was carried out following the method proposed by Steibel, Poletto, Coussens, and Rosa (2009), which consists of the analysis of cycles to threshold values (C_p), for the targets and endogenous genes using a linear mixed model. The following model was used for analyzing the joint expression of the target and housekeeping genes in muscle:

$$y_{gijkr} = TG_{gi} + L_{gj} + B_{gjk} + D_{ijk} + e_{gijkr}$$

where $y_{gijkr} = -log_2\left(E_g^{-Cp_{gijkr}}\right) y_{gijk} = log_2\left(E_{gijk}^{-C_{gijk}}\right)$, *Eg* is the efficiency of the PCR of *g*th gene, C_{pgijkr} is the value obtained from the thermocycler software for the *g*th gene from the *r*th replicate in a sample collected from the *k*th animal of the *j*th litter fed with the *i*th dietary treatment, TG_{gi} is the specific effect of the *i*th dietary treatment on the expression of gene *g*th, L_{gj} and B_{gjk} are specific random effects of the *j*th full-sib family and the *k*th pig on the expression of

gene gth, D_{ijk} is a random sample-specific effect common to all the genes, and e_{gijkr} is a residual effect.

To test differences in the expression rate of genes of interest ($diff_{TG}$) between classes normalized by the housekeeping genes, different comparisons were performed between the respective estimates of *TG* levels. Significance of $diff_{TG}$ estimates was determined with the t statistic. To obtain fold change (*FC*) values from the estimated $diff_{TG}$ values, the following equation was applied: $FC = 2^{-diff_{TG}}$. $\mathbf{FC = 2^{-diff_{TG}}}$ Asymmetric 95% confidence intervals (CI) were calculated for each *FC* value by using the standard error (SE) of the estimated difference: 95% CI from $\mathbf{2^{[-(diff]_{TG}+1.96\ x\ SE)}}$ $\mathbf{FC_{UP} = 2^{[-(diff]_{TG}-1.96\ x\ SE)}}$ $2^{(-\mathrm{diff_{TG}}+1.96\mathrm{xSE})}$

3.4.4- Results

Carcass traits and performance parameters

Dietary vitamin A restriction had no effect ($P > 0.05$) on the studied carcass traits (carcass weight, carcass length, shoulder and ham weight, ham length and backfat thickness) at early growing or finishing periods, as shown in Table 3. Performance parameters were also evaluated on the overall period; there were no differences on average daily gain (0.52 ± 0.01 and 0.52 ± 0.02 kg/d in CONTROL and VAR animals, respectively; $P = 0.75$), average daily feed intake (2.09 ± 0.9 and 2.08 ± 0.08 kg feed/d in CONTROL and VAR animals, respectively; $P = 0.99$) and feed conversion ratio (4.02 ± 0.18 and 4.01 ± 0.13 kg gain/kg in CONTROL and VAR animals, respectively; $P = 0.99$)

Table 3: Carcass characteristics according to dietary vitamin A treatment and productive phase (early growth or finishing).

		Treatment		RMSE[a]	*P* - value
		CONTROL	VAR[c]		
Carcass weight	Growth[d]	28.24	27.57	2.61	0.5941
	Finishing[e]	122.75	124.41	5.38	0.1555
Ham weight	Growth	3.61	3.50	0.35	0.5032
	Finishing	14.60	13.79	0.85	0.4653
Shoulder weight	Growth	2.39	2.41	0.28	0.8881
	Finishing	8.70	8.72	0.43	0.3979
Backfat thickness	Growth	16.30	16.00	0.87	0.8340
	Finishing	63.50	62.50	4.46	0.7163
Carcass lenght (cm)	Finishing	81.30	80.55	1.05	0.4486
Ham lenght (cm)	Finishing	44.65	43.85	0.75	0.2285
Ham perimeter (cm)	Finishing	77.15	75.60	1.43	0.1759

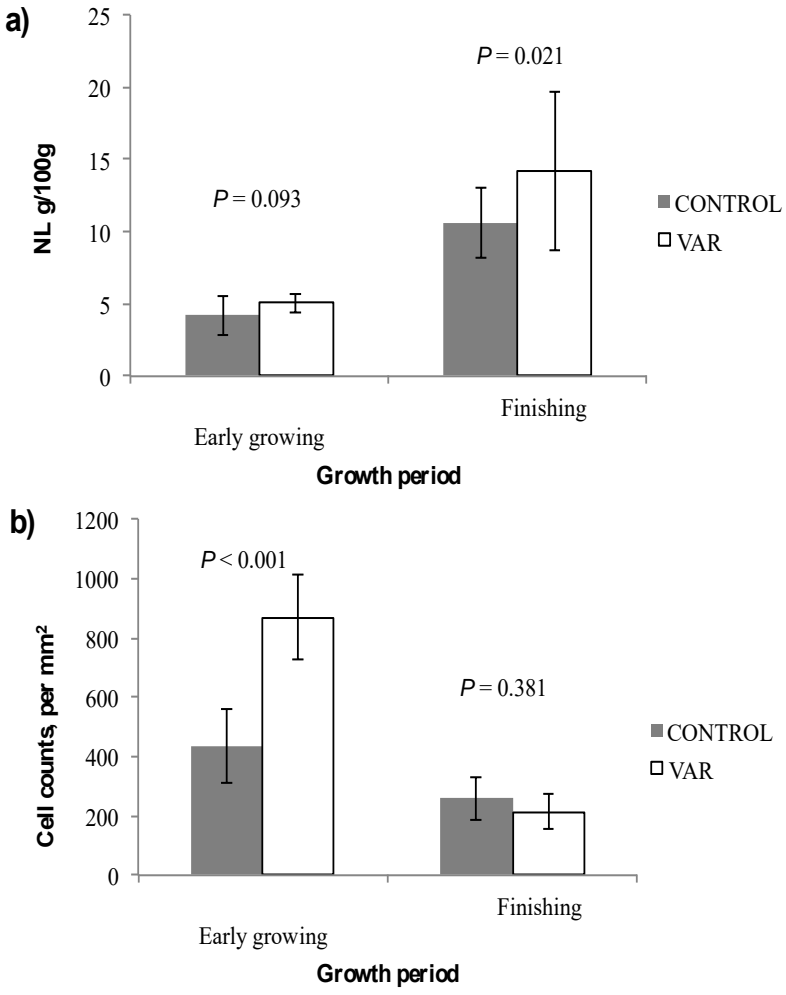
[a]RMSE = root-mean-square error.
[b]CONTROL = CONTROL diet. 10,000 IU vitamin A/kg feed supplementation.
[c]VAR = Vitamin A restricted diet. 0 IU vitamin A/kg feed supplementation.
[d]Growth = Early growth phase; carcass traits measured at 35.8 kg LW.
[e]Finishing = Finishing phase; carcass traits measured at 158 kg LW.

Neutral lipids and preadipocyte content of intramuscular fat

Neutral lipids content in the IMF did not show any difference at early growing (4.2 ± 1.3 vs 5.1 ± 0.6 g/100 g tissue for CONTROL and VAR groups, respectively; $P = 0.09$). The Iberian pigs from VAR group showed a higher content in NL than those from the CONTROL group at finishing (Fig 1a) (10.6 ± 2.4 vs 14.2 ± 3.4 g/100 g tissue for CONTROL and VAR groups, respectively $P = 0.02$).

In an effort to identify changes in the IMF induced by dietary vitamin A level, we conducted immunohistochemical analysis to identify and quantify preadipose cells. The number of preadipocytes showed significant differences between diets at early growing but we did not find any difference at finishing. In young animals the number of preadipose cells was higher in the VAR group than in the CONTROL group (868 ± 143 cells/mm^2 vs 502 ± 211 cells/mm^2, $P < 0.001$) (Fig 1b).

Figure 1. Effect of vitamin A restriction on: a) Neutral Lipids from *Longissimus thoracis* muscle (g/100 g total fatty acids) and b) Preadipocyte number of Iberian pigs (cell counts/mm^2) slaughtered at early growing and finishing.
Black bars = CONTROL group; pigs received a 10,000 IU vitamin A supplementation /kg diet; Open bars = VAR group; pigs received a 0 IU vitamin A supplementation/kg diet.



Fatty acids composition of Longissimus thoracis muscle

Fatty acid profiles were analyzed in LT muscle samples at early growing and finishing (Table 4 and 5, respectively). At the end of the early growth period, no effect of dietary treatment was observed in NL, but PL in CONTROL group showed higher C16:1 n-7, C18:0, C18:1 n-9, C20:0, C20:1 n-9, SFA and MUFA, and a lower C18:2 n-6, C20:4 n-6, C20:5 n-3, C22:4 n-6, PUFA, ∑n-6 and ∑n-3 concentrations than those fed the VAR diet.

Table 4: Fatty acid composition of *Longissimus thoracis* muscle (g/100 g total fatty acids) at the early growth phase.

	Neutral lipids				Polar lipids			
	Feeding Treatments		RMSE[a]	P-value	Feeding Treatments		RMSE	*P* -value
	CONTROL[b]	VAR[c]			CONTROL	VAR		
C14:1	1.41	1.40	0.09	0.938	2.36	2.31	0.18	0.602
C16:0	24.94	24.25	1.50	0.344	23.34	22.72	0.79	0.117
C16:1 n-9	0.32	0.30	0.06	0.402	0.33	0.33	0.05	0.727
C16:1 n-7	3.59	3.61	0.35	0.914	1.03	0.87	0.14	0.026
C17:0	0.35	0.33	0.04	0.496	0.75	0.72	0.10	0.479
C17:1	0.48	0.48	0.07	0.990	0.88	0.99	0.23	0.332
C18:0	14.16	13.68	0.64	0.131	11.12	10.23	0.87	0.045
C18:1 n-9	40.39	40.80	1.59	0.587	16.67	14.18	2.10	0.023
C18:1 n-7	2.92	2.82	0.31	0.483	2.79	2.78	0.18	0.924
C18:2 n-6	7.65	8.35	1.38	0.296	25.13	27.29	2.17	0.050
C18:3 n-3	0.43	0.48	0.07	0.116	0.89	0.86	0.12	0.688
C18:4 n-3	0.17	0.16	0.02	0.408	0.11	0.11	0.03	0.804
C20:0	0.17	0.18	0.03	0.592	0.65	0.33	0.23	0.008
C20:1 n-9	0.83	0.82	0.07	0.911	0.35	0.27	0.07	0.026
C20:2 n-6	0.40	0.41	0.05	0.691	0.45	0.54	0.11	0.109
C20:3 n-6	0.16	0.17	0.04	0.527	0.93	1.01	0.12	0.196
C20:4 n-6	0.93	1.04	0.31	0.486	7.65	8.79	0.73	0.004
C20:5 n-3	0.05	0.06	0.02	0.079	0.34	0.39	0.04	0.009
C22:1 n-9	0.09	0.07	0.03	0.260	0.10	0.06	0.05	0.085
C22:4 n-6	0.23	0.23	0.05	0.857	1.50	1.68	0.18	0.049
C22:5 n-3	0.14	0.16	0.03	0.190	1.07	1.92	1.08	0.112
C22:6 n-3	0.07	0.05	0.03	0.136	0.48	0.52	0.13	0.558
SFA[d]	39.62	38.45	1.62	0.141	36.55	34.46	1.51	0.010
MUFA[e]	50.02	50.30	1.91	0.761	24.56	21.90	2.03	0.013
PUFA[f]	10.35	11.25	1.81	0.308	38.89	43.63	2.93	0.003
Σn3	0.94	1.00	0.11	0.229	2.94	4.01	1.05	0.046
Σn6	9.42	10.25	1.71	0.317	35.95	39.63	2.67	0.010
Σn6/Σn3	9.96	10.23	0.97	0.565	12.31	10.78	2.30	0.178
C18:1/C18:0	3.06	3.20	0.17	0.115	1.75	1.66	0.15	0.220
MUFA/SFA	1.26	1.31	0.10	0.275	0.67	0.64	0.06	0.165

[a]RMSE = root-mean-square error.
[b]CONTROL = CONTROL diet. 10,000IU vitamin A/kg feed supplementation.
[c]VAR = Vitamin A restricted diet. 0 IU vitamin A/kg feed supplementation.
[d]SFA (C12:0+C14:0+C16:0+C17:0+C18:0+C20:0); sum of saturated fatty acids.
[e]MUFA (C16:1n-9+C16:1n-7+C17:1+C18:1n-9+C18:1n-7+C20:1n-9); sum of monounsaturated fatty acids.
[f]PUFA (C18:2n-6+C18:3n-3+C20:3n-9+C20:4n-6); sum of polyunsaturated fatty acids.

Table 5: Fatty acid composition of *Longissimus thoracis* muscle (g/100g total fatty acids) at finishing.

	Neutral lipids				Polar lipids			
	Feeding Treatments		RMSE[a]	P-value	Feeding Treatments		RMSE	*P* - value
	CONTROL	VAR[c]			CONTRO	VAR		
C14:1	1.57	1.31	0.21	0.014	2.66	2.80	0.49	0.529
C16:0	26.06	24.06	1.10	0.001	19.55	18.6	1.76	0.275
C16:1 n-9	0.17	0.17	0.03	0.752	0.12	0.10	0.04	0.291
C16:1 n-7	4.11	3.98	0.53	0.600	0.82	0.82	0.26	0.950
C17:0	0.14	0.16	0.04	0.465	0.42	0.43	0.05	0.764
C17:1	0.19	0.21	0.04	0.141	1.93	2.24	0.60	0.272
C18:0	11.58	10.61	0.75	0.011	8.92	7.78	2.02	0.222
C18:1 n-9	48.12	51.29	1.34	<.0001	16.79	16.0	3.44	0.644
C18:1 n-7	3.35	3.41	0.36	0.757	3.27	2.96	0.49	0.175
C18:2 n-6	2.85	3.02	0.53	0.499	27.16	29.5	4.05	0.199
C18:3 n-3	0.17	0.19	0.04	0.294	0.36	0.46	0.04	<.0001
C18:4 n-3	0.10	0.12	0.01	0.043	0.06	0.06	0.02	0.612
C20:0	0.17	0.15	0.02	0.063	0.40	0.27	0.28	0.318
C20:1 n-9	0.89	0.85	0.11	0.422	0.38	0.34	0.26	0.756
C20:2 n-6	0.19	0.21	0.06	0.497	0.50	0.51	0.06	0.810
C20:3 n-6	0.05	0.04	0.01	0.094	1.05	1.11	0.15	0.407
C20:4 n-6	0.13	0.12	0.02	0.145	11.20	10.9	1.71	0.735
C20:5 n-3	ND[d]	ND	ND	ND	0.35	0.43	0.10	0.109
C22:1 n-9	0.02	0.03	0.01	0.485	0.43	0.40	0.24	0.792
C22:4 n-6	0.05	0.04	0.01	0.107	1.36	1.38	0.23	0.853
C22:5 n-3	0.07	0.06	0.06	0.816	1.15	1.23	0.20	0.413
C22:6 n-3	ND	ND	ND	ND	0.42	0.48	0.18	0.454
SFA[e]	37.96	34.97	1.02	<.0001	29.72	27.5	3.86	0.223
MUFA[f]	58.44	61.24	1.00	<.0001	26.16	25.5	2.96	0.650
PUFA[g]	3.60	3.79	0.64	0.537	44.12	46.9	5.98	0.311
Σn3	0.34	0.36	0.07	0.471	2.34	2.66	0.35	0.055
Σn6	3.27	3.43	0.59	0.567	41.27	43.5	5.67	0.390
Σn6/Σn3	9.99	9.50	1.40	0.460	17.72	16.5	2.32	0.270
C18:1/C18:0	4.46	5.18	0.32	<.0001	2.33	2.48	0.38	0.379
MUFA/SFA	1.54	1.75	0.07	<.0001	0.89	0.93	0.10	0.339

[a]RMSE = root-mean-square error.
[b]CONTROL = CONTROL diet. 10,000 IU vitamin A/kg feed supplementation.
[c]VAR = Vitamin A restricted diet. 0 IU vitamin A/kg feed supplementation.
[d]ND = not detectable.
[e]SFA (C12:0+C14:0+C16:0+C17:0+C18:0+C20:0); sum of saturated fatty acids.
[f]MUFA (C16:1n-9+C16:1n-7+C17:1+C18:1n-9+C18:1n-7+C20:1n-9); sum of monounsaturated fatty acids.
[g]PUFA (C18:2n-6+C18:3n-3+C20:3n-9+C20:4n-6); sum of polyunsaturated fatty acids.

Regarding the fatty acid composition in the finishing period, main differences were observed in the NL fraction. The VAR animals showed lower SFA and higher MUFA concentrations, mainly due to lower C16:0 and C18:0 acids and higher C18:1 n-9 content, respectively. Pigs fed the VAR diet showed also higher C18:4 n-3 and lower C14:1. In the PL fraction, only a higher C18:3 n-3 content and a trend ($P = 0.06$) for higher n-3 fatty acids were observed in the VAR group.

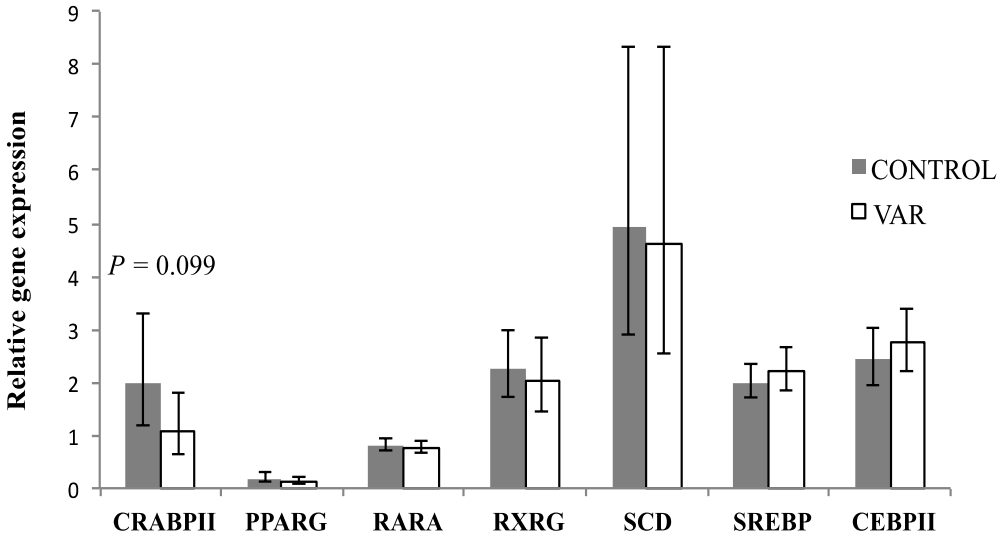
No effects of Vitamin A restriction were observed on desaturase index (C18:1/C18:0 and MUFA/SFA) at the end of the early growth period in the NL or PL fractions. However we found higher desaturation index in the NL of IMF at finishing in Iberian pigs fed the VAR diet ($P < 0.0001$).

Expression of candidate genes

Figure 2 shows relative gene expression values of both dietary treatments in LT muscle. *CRABPII* gene expression tended to be higher in CONTROL than in the VAR group ($P = 0.099$), with 1.85-fold increase.

However, no differences for *PPARG, RARA, RXRG, SCD, SREBP* or *CEBPB* genes expression were observed.

Figure 2. Relative expression values of candidate genes of CONTROL and VAR groups in *Longissimus thoracis* muscle at early growing.



CEBPB: CCAAT/enhancer binding protein β
PPARG: Peroxisome proliferator-activated receptor G
SREBP-1c: Sterol regulatory binding transcription factor 1
RARA: Retinoic acid receptor α
RXRG: Retinoid X receptor G
CRABP2: Cellular retinoic acid binding protein 2
SCD: Stearoyl-CoA desaturase

3.4.5- Discussion

Carcass traits and performance parameters

Vitamin A restriction did not affect carcass characteristics at either early growing or finishing periods, in agreement with Olivares et al. (2009a,b) and D'Souza, Pethick, Dunshea, Pluske, and Mullan (2003). It is noteworthy that vitamin A restriction applied from so early at growth stages

did not affect growth and body development, whereas in the study of Olivares et al. (2009a), vitamin A was restricted only during the last 5 weeks of life, in the fattening phase.

Preadipocyte and Neutral lipids content of intramuscular fat

To our knowledge, this is the first study reporting the preadipocyte number in LT muscle; a large number of preadipocytes were observed in muscle tissue at growing, and their number decreased in both treatments over time. Adipocyte differentiation is an extremely complex process and meat-animal-derived preadipocytes and adipocytes studies have shown that conversion and differentiation processes are not necessarily the same as those observed in rodent systems or in cell lines (Hausman, Basu, Du, Fernyhough-Culver, & Dodson, 2014).

Lipid accumulation is the result of both hyperplasia and hypertrophy. Hyperplasia leads to an increase in adipose cell number during growth stages that may be related to higher IMF content in adult animals (Hausman et al., 2014). An effect of dietary vitamin A level on hyperplasia ability was observed in this study in Iberian pigs at early growing; samples from LT muscle showed significantly more preadipocytes in the VAR group ($P < 0.001$). However, hypertrophy is a posterior event and no effect on NL content was observed at that early stage, although we found higher NL in VAR group at finishing. Neutral lipids fraction represents 60-70% of total fatty acids in the IMF (Tous et al., 2013) and is mainly composed by reserve lipids and thus, it can be used as an estimator of IMF. The increase in NL content at 158 ± 7 kg LW may be a consequence of a higher potential for lipid deposition in the VAR group (Fig 1a). The presence of a higher number of preadipocytes in early growing pigs can indicate greater potential to differentiate and accumulate lipids over the experimental period, since IMF accumulation is dependent on the increase in the number of adipocyte differentiations from preadipocytes. This fact is an important regulatory step in the deposition of marbling; thus, higher number of preadipose cell counts (associated with higher potential for lipid deposition) in muscle tissue during growth stages may be related to higher IMF content in adult animals, as reportedd by Hausman et al. (2014). This is consistent with the higher NL content observed in de VAR pigs at finishing.

In pigs, the research from Quiniou, Richard, Mourot, and Etienne (2008) showed that preadipocyte and adipocyte cells may change depending on dietary fat in the sow diets at an early age (10.2 kg LW) in subcutaneous adipose tissue and in the IMF without affecting slaughter weight or carcass lean meat content.

It has been reported that vitamin A restriction may influence specifically the IMF cell number (the cellularity of the IMF) without influencing the subcutaneous depot in steers (Gorocica-Buenfil, Fluharty, Reynolds, & Loerch, 2007b). In agreement with these authors, backfat thickness was not affected by long-term vitamin A restriction in our experiment. On the other hand, the effect of vitamin A level on IMF content is still controversial. In accordance to our results, Olivares et al. (2011), in a study in lean pigs from 55.8 kg LW to 125 kg LW (approx. 11 weeks of restriction)

found that pigs fed low dietary vitamin A levels (1,300 IU) had a higher IMF content in LT muscle when compared with a CONTROL group fed 13,000 IU, 10-fold higher than the standard recommended level (NRC, 1998). However the same authors in a previous study with high dietary vitamin A supplementation (100,000 IU vs. 0 IU) found different responses to vitamin A supplementation depending on the genotype (Olivares et al., 2009b). In Duroc pigs, these authors found a 20% increase in the IMF content of LT muscle in those animals fed the 100,000 IU vitamin A enriched diet, while no effect was observed in Landrace x Large White for a restriction time of 8 weeks and 114.5 kg LW at slaughter. In our study the restriction time for Iberian pigs was two months at 35.8 ± 3.1 kg LW (early growing) and nine months for the pigs slaughtered at 158 ± 7 kg LW (finishing). According to these results, the effect of dietary vitamin A on IMF content might depend on restriction or supplementation duration, age at the beginning of the restriction or supplementation treatment and pig genotype. Thus, further studies are needed to determine the effect of different dietary vitamin A restriction levels and times on pig breeds of interest. Similarly, the results obtained in ruminant animals are not consistent (Gorocica-Buenfil et al., 2007b; Siebert et al., 2006).

Fatty acids concentration of Longissimus thoracis muscle

The higher PUFA content observed at early growing could be related to a preferential mitochondrial transport and beta-oxidation for polyunsaturated rather than for saturated fatty acids in Iberian pigs fed the vitamin A supplemented diet. There is a lack of information on the influence of the dietary vitamin A inclusion level on enzyme activities. Sanz, Lopez-Bote, Menoyo, and Bautista (2000) and Shimomura, Tamura, and Suzuki (1990) observed that dietary saturated fat decreases beta-oxidation but the effect of other nutrients on beta-oxidation remains unclear.

Siebert et al. (2006) found that the desaturation index was inversely related to plasma vitamin A levels in Angus steers. Jeyakumar, Vajreswari, and Giridharan (2008) found that feeding a high level of vitamin A led to an increase of SCD activity in lean rats, but this effect was not observed in obese rats. Olivares et al. (2011) reported a decrease in the MUFA/SFA ratio in backfat but not in the IMF in pigs fed a diet with1,300 IU vitamin A for eleven weeks prior to slaughter. These results are not in agreement with those obtained in the present experiment since a higher desaturation index (MUFA/SFA) was observed in the NL of IMF from pigs fed the VAR diet at finishing. However it should be noticed that in the present experiment the animals were subjected to vitamin A restriction for a total of nine months, and they were of an obese genotype, whereas in the experiment of Olivares et al. (2011), vitamin A was restricted for just 11 weeks and the animals were of a lean genetic line (Large White x Landrace). This suggests that the effect of vitamin A on fatty acids profile might be dependent on vitamin A inclusion levels, genotype and length of experimental period, in accordance to the results obtained in LN fraction content.

Expression of candidate genes

To understand the role of vitamin A in preadipocyte differentiation, a gene expression analysis was carried out. Retinoic acid is well known as a potent transcription regulator, and some genes regulated by RA are involved in adipocyte differentiation and RA signalling, like nuclear receptors or binding proteins (Bonet et al., 2003; Noy, 2013). We investigated gene expression in young (35.8 kg) pigs, when genes related to adipocyte differentiation are expected to be more active and thus may have a greater response potential. However, we found no difference in gene expression in this stage. A possible reason of this lack of changes in gene expression after two months of treatment may be because the effects of dietary vitamin A restriction need a bit longer treatment to be detectable. Vitamin A is a liposoluble vitamin that accumulates in body tissues and thus body depots depletion is needed prior to changes in retinol homeostasis. A trend was observed for greater expression of the *CRABPII* gene in the CONTROL group. This gene codes for an intracellular protein that binds RA in the cytoplasm and transports it into the nucleus, thus, increasing its binding into nuclear receptors (Dong, Ruuska, Levinthal, & Noy, 1999). Previous studies have shown the influence of this protein in adipocyte development. Berry, Soltanian, and Noy (2010) confirmed that downregulation of *CRABPII* is a critical component in the differentiation process and that this protein markedly sensitizes preadipocytes to RA-induced inhibition of adipogenesis. Thus, our finding may suggest that adipogenesis is a more active process in the VAR group; this fact together with an increased number of preadipose cells in this group may lead to a greater development of the fat compartment in adult animals, as shown in the vitamin A restricted animals at finishing.

3.4.6- Conclusion and implications

Decreasing added vitamin A in feed from two to four months of age caused an increase in the number of preadipocytes and suggestive downregulates the *CRABPII* gene. In addition, vitamin A restriction for 9 months caused a pronounced increase of total NL, monounsaturated fatty acids and a decrease of saturated fatty acids in NL fraction of IMF. This change in fatty acids profile has a positive effect on costumer´s health.

This approach is an easy and no cost-increasing strategy to increase the lipid content in muscle of Iberian pigs with no detrimental effects on carcass traits. The greater lipid content would lead to an increase in meat quality, which is highly appreciated in meat products obtained from this breed. However, further studies on different added dietary vitamin A level (0, NRC recommended levels, commercial levels) would improve the knowledge about this topic.

Acknowledgements

This research was supported by Comisión Interministerial de Ciencia y Tecnología (CICYT-AGL2010-21991-C03-01) and Comunidad de Madrid (MEDGAN S2013/ABI-2913 MEDGAN-CM). We are grateful to the staff of "El Dehesón del Encinar" for their invaluable help an also to De la Torre, I for technical support.

3.5 CAPITULO 5: La restricción prolongada de vitamin A mejora los parámetros de calidad de carne y modifica la expression génica en cerdos ibéricos.

Long term vitamin A restriction improves meat quality parameters and modifies gene expression in Iberian pigs

M. Ayuso; A. Fernández; B. Isabel; A. Rey; R. Benítez; A. Daza; C. J. López-Bote; C. Óvilo.

J Anim Sci. 2015; 93 (6): 2730-44.

Published June 25, 2015

Long term vitamin A restriction improves meat quality parameters and modifies gene expression in Iberian pigs[1]

M. Ayuso,*[2] A. Fernández,† B. Isabel,* A. Rey,* R. Benítez,† A. Daza,‡ C. J. López-Bote,* and C. Óvilo†

*Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, E-28040 Madrid, Spain; †Departamento de Mejora Genética Animal, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Ctra. Coruña Km. 7.5, E-28040 Madrid, Spain; and ‡Departamento de Producción Animal, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid, E-28040 Madrid, Spain

ABSTRACT: Vitamin A is a key regulator of gene expression, influencing adipogenesis and lipid metabolism in animal tissues. This experiment was conducted to assess the effect of dietary vitamin A level and administration time on productive traits, intramuscular fat (IMF) content in ham muscles, tissue fatty acid composition, and expression of a panel of adipogenic and lipogenic candidate genes in Iberian pigs. Sixty piglets of 16.3 kg (SD = 2.5 kg) live weight (LW) were either fed a vitamin A–enriched diet (10,000 IU vitamin A/kg; CONTROL, $n = 20$) or a diet without supplemented vitamin A, applied from 16.3 kg (SD = 2.5 kg; early restriction group, ER, $n = 20$) or from an average weight of 35.8 kg (SD = 3.1 kg; late restriction group, LR, $n = 20$). Two slaughters were performed when pigs reached the averaged weights of 101.4 (SD = 4.1 kg) and 157.9 kg LW (SD = 7 kg) and samples from liver, heart, and backfat were obtained in both sacrifice times. In addition, ham subcutaneous fat and *Semimembranosus* (SM) and *Biceps Femoris* (BF) muscles were sampled at the last sacrifice. Dietary vitamin A level produced no effect on carcass traits in any of the harvests, while a small effect was observed on fatty acid composition in backfat at 101.4 kg LW. However, at 157.9 kg LW, the ER and LR groups showed higher MUFA content and lower SFA content in backfat, ham fat, and IMF ($P < 0.01$). In IMF, a decrease in n-6/n-3 PUFA ratio was observed in the restricted groups ($P < 0.005$). Intramuscular fat content in SM muscle was greater ($P < 0.05$) in the ER group than in the CONTROL and LR groups, while no difference was detected in BF muscle. Little effect of dietary vitamin A was observed in liver. Regarding changes in gene expression, *ACSL4*, *CEBPB*, and *IGF1* genes were upregulated ($P < 0.0001$, $P < 0.0001$, and $P < 0.05$, respectively) in the ER group in hepatic tissue, whereas *CRABPII* and *SCD* genes were upregulated ($P < 0.05$) in the same group in adipose tissue. On the other hand, *RXRG* was downregulated ($P < 0.05$) in the ER group in adipose tissue. Results found in this experiment show that long-term restriction of dietary vitamin A has a positive effect on nutritional and sensorial parameters of ham meat. Moreover, gene expression results were consistent with the vitamin A transcriptional regulation of adipogenesis and lipogenesis and with the changes observed in meat and fat composition.

Key words: fatty acid composition, gene expression, Iberian pig, meat quality, vitamin A

J. Anim. Sci. 2015.93:2730–2744
doi:10.2527/jas2014-8573

INTRODUCTION

Intramuscular fat (**IMF**) content and composition are determinant factors for meat quality (Wood et al., 2008). Meat flavor, tenderness, and juiciness are mainly dependent on fat presence and compo-

[1]The authors are grateful to CIA Dehesón del Encinar (Toledo). Technical support from I. De la Torre is also acknowledged. This research was supported by Comisión Interministerial de Ciencia y Tecnología (AGL2010-21991-C03).

[2]Corresponding author: mayuso@ucm.es

Received October 2, 2014.
Accepted April 2, 2015.

3.5.1- Abstract

Vitamin A is a key regulator of gene expression, influencing adipogenesis and lipid metabolism in animal tissues. This experiment was conducted to assess the effect of dietary vitamin A level and administration time on productive traits, intramuscular fat (IMF) content in ham muscles, tissue fatty acid composition and expression of a panel of adipogenic and lipogenic candidate genes in Iberian pigs. Sixty piglets of 16.3 kg (SD = 2.5 kg) live weight (LW) were either fed a vitamin A-enriched diet (10,000 IU vitamin A/kg) (CONTROL, n = 20) or a diet without supplemented vitamin A, applied from16.3 kg (SD = 2.5 kg) (early restriction group, ER, n = 20), or from an average weight of 35.8 kg (SD = 3.1 kg) (late restriction group, LR, n = 20). Two slaughters were carried out when pigs reached the averaged weights of 101.4 (SD = 4.1 kg) and 157.9kg LW (SD = 7 kg) and samples from liver, heart and backfat were obtained in both sacrifice times. In addition, ham subcutaneous fat and *Semimembranosus* (SM) and *Biceps Femoris* (BF) muscles were sampled at the last sacrifice. Dietary vitamin A level produced no effect on carcass traits in any of the harvests whilst a small effect was observed on fatty acid composition in backfat at 101.4 kg LW. However, at 157.9kg LW, the ER and LR groups showed higher MUFA content and lower SFA content in backfat, ham fat and IMF ($P < 0.01$). In IMF, a decrease in n-6/n-3PUFA ratio was observed in the restricted groups ($P < 0.005$). Intramuscular fat content in SM muscle was greater ($P < 0.05$) in the ER group than in the CONTROL and LR groups whilst no difference was detected in BF muscle. Little effect of dietary vitamin Awas observed in liver. Regarding changes in gene expression, *ACSL4*, *CEBPB* and *IGF1* genes were upregulated ($P < 0.0001$, $P < 0.0001$ and $P < 0.05$, respectively) in the ER group in hepatic tissue, whereas *CRABPII* and *SCD* genes were upregulated ($P < 0.05$) in the same group in adipose tissue. On the other hand, *RXRG* was downregulated ($P < 0.05$) in the ER group in adipose tissue. Results found in this experiment show that long term restriction of dietary vitamin A has a positive effect on nutritional and sensorial parameters of ham meat. Moreover, gene expression results were consistent with the vitamin A transcriptional regulation of adipogenesis and lipogenesis, and with the changes observed in meat and fat composition.

Keywords: fatty acid composition, gene expression, Iberian pig, meat quality, vitamin A.

3.5.2- Introduction

Intramuscular fat (**IMF**) content and composition are determinant factors for meat quality (Wood et al., 2008). Meat flavour, tenderness and juiciness are mainly dependent on fat presence and composition and several nutritional strategies have been developed to improve these properties (Wood et al., 2008). It has been shown that the energy intake or the protein/calorie ratio affect carcass fat and IMF content, and that dietary fatty acids affect adipose tissue fatty acid profiles in pigs (Witte et al., 2000; Gatlin et al., 2002; Suarez-Belloch et al., 2013). More recent research has focussed on micronutrients content, such as conjugated linoleic acid (CLA (Cordero et al., 2010) and vitamin A (Olivares et al., 2009a, 2009b, 2011).

Minimum vitamin A recommended dietary levels have been established as 1,317 IU/kg diet by NRC (National Research Council, 2012), but practical formulation tends to include higher levels of vitamin A (Fraga and Villamide, 2000). It has been described that vitamin A negatively affects IMF content in livestock (Oka et al., 1998; Olivares et al., 2011), although contrary results have also been reported (Daniel et al., 2004; Arnett et al., 2007; Olivares et al., 2009a). Olivares et al. (2009b) observed that the effect of dietary vitamin A level on IMF is dependent on pig genotype. D'Souza et al. (2003) reported a positive effect of dietary vitamin A restriction on IMF content in lean pigs. Thus, the effect of vitamin A in pigs remains unclear and there are no previous studies assessing these effects in high-fat breeds, such us the Iberian pig breed. Besides, no research has been carried out on the effect of timing of dietary vitamin A withdrawal. Therefore, the objectives of the present study were: 1) to study the effect of different periods of dietary vitamin A restriction on carcass traits, IMF content and fatty acid composition in different tissues from Iberian pigs and; 2) to elucidate how this effect is mediated by changes at the gene expression and enzyme activity levels.

3.5.3- Material and methods

Animals and diets

Animal manipulations were performed in compliance with the regulations of the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used in experimentation. The experiment was specifically assessed and approved (report CEEA 2010/003) by the Spanish National Institute for Agricultural and Food Research and Technology (INIA) Committee of Ethics in Animal Research. The trial was conducted at CIA Dehesón del Encinar (Oropesa, Toledo, Spain).

Sixty castrated male (Torbiscal Iberian) piglets from 25 litters (full-sib families) were used. Piglets were weaned at 1 month of age and fed a commercial starter diet containing 10,000 IU vitamin

A/kg diet for 1 month. At the age of 2 months, with an average weight of 16.3 kg (SD = 2.5 kg), piglets were randomly allocated into 2 groups and housed in pairs. One group (n = 40) was fed a vitamin A-enriched starter diet (10,000 IU vitamin A/kg diet) and the other group (n = 20) received a starter diet formulated with no vitamin A added in the premix (early restriction group, **ER**). At the age of 4 months and an average weight of 35.8 kg (SD = 3.1 kg), 20 piglets from the first group were changed to the vitamin A restricted diet (0 UI vitamin A/ kg diet) (late restriction group, **LR**) and the remaining 20 continued to receive the vitamin A enriched diet (**CONTROL**). At 32.2 kg (SD = 4.5 kg) live weight (**LW**) all 60 pigs were fed the corresponding vitamin A-enriched and vitamin A-restricted growing diets and this level of added vitamin A in the diet was maintained throughout the growing and finishing period. When pigs reached 101.4 kg LW (SD = 4.1 kg), they were housed individually and changed to a fattening diet, until slaughter at 157.9 kg (SD = 7 kg) LW. Pigs were fed 3.5% live weight feed restriction from 2 to 4 months of age, 3% live weight feed restriction from 4 to 8 months of age and 2.5% live weight feed restriction from 8 to 11 months of age. Pigs had *ad libitum* access to water.

Ingredients and chemical composition of experimental diets are shown in Table 1. Diets were formulated according to general guidelines proposed by De Blas et al. (2013) for Iberian pigs.

Table 1: Diet composition and calculated analysis (g/kg, as-fed basis)

	Starter (<32.2 kg)		Growth (32.2- 101.4		Fattening (101.4-	
	C[1]	R[2]	C	R	C	R
Ingredients						
Barley	280.0	280.0	500.0	500.0	453.7	453.7
Soybean meal (440g	155.1	155.1	169.4	169.4	75.4	75.4
Wheat	250.0	250.0	290.3	290.3	300.0	300.0
Soybean protein (650 g CP/kg)	25	25				
Corn	194.9	194.9				
Whey powder, sweet	25.0	25.0				
Lard	17	17	10.0	10.0	20.0	20.0
Full fat soybean toasted	20.0	20.0				
High oleic sunflower seed					120.0	120.0
Calcium carbonate	5.4	5.4	8.2	8.2	8.2	8.2
Dicalcium phosphate	13.6	13.6	12.0	12.0	12.0	12.0
Mineral and vitamin	4.0	0	4.0	0	4.0	0
Mineral and vitamin	0	4.0	0	4.0	0	4.0
Salt	4.0	4.0	4.5	4.5	4.5	4.5
L-Lysine (500 g/kg)	4.0	4.0	1.6	1.6	2.2	2.2
Methionine-OH	1.4	1.4				
L-Threonine	0.6	0.6				
Calculated analysis[5]						
Net energy, MJ/kg	10.0	10.0	9.5	9.5	10.4	10.4
Dry matter, g/kg	895.0	895.0	989.8	989.8	990.3	990.3
Ash , g/kg	48.0	48.0	49.2	49.2	46.6	46.6
Crude protein, g/kg	178.2	178.2	158.0	158.0	135.0	135.0
Crude fat, g/kg	41.9	41.9	26.8	26.8	82.3	82.3
Crude fibre, g/kg	35.7	35.7	40.3	40.3	55.2	55.2
Added retinol, IU/kg feed[6]	10000	0	10000	0	10000	0

[1]CONTROL = Diet was enriched with 10,000 IU of vitamin A/kg diet as retinyl acetate

[2]Restriction = Diet was formulated to contain 0 IU of vitamin A/kg diet as retinyl acetate

[3]Vitamin-mineral premix provided per kg of feed; Vitamin A, 10,000 IU; Vitamin D3, 2,000 IU; Vitamin E, 26.7 mg; Vitamin B1, 1.3 mg; Vitamin B2, 4.0 mg; vitamin B12, 0.020 mg; Vitamin B6, 1.3 mg; Calcium pantothenate, 13.3 mg; Nicotinic acid, 20 mg; Biotin, 0.1 mg; Folic acid, 0.1 mg; Vitamin K3, 2 mg; Fe, 133.3 mg; Cu, 26.7 mg; Co, 0.30 mg; Zn, 133.3 mg; Mn, 76.7 mg; I, 1.3 mg; Se, 0.30 mg; Ethoxyquin, 150 mg.

[4]Vitamin-mineral premix provided per kg of feed; Vitamin A, 0 IU; Vitamin D3, 2,000 IU; Vitamin E, 26.7 mg; Vitamin B1, 1.3 mg; Vitamin B2, 4.0 mg; vitamin B12, 0.020 mg; Vitamin B6, 1.3 mg; Calcium pantothenate, 13.3 mg; Nicotinic acid, 20 mg; Biotin, 0.1 mg; Folic acid, 0.1 mg; Vitamin K3, 2 mg; Fe, 133.3 mg; Cu, 26.7 mg; Co, 0.30 mg; Zn, 133.3 mg; Mn, 76.7 mg; I, 1.3 mg; Se, 0.30 mg; Ethoxyquin, 150 mg.

[5]According to Fundación Española Desarrollo Nutrición Animal (2010) (supplied per kg of diet).

[6]Calculated retinol values refer to the retinol added within the vitamin-mineral premix, but not to dietary contents. Dietary vitamin A content was not 0 in restricted diets due to the carotenoids content in raw materials (Giambanelli et al., 2013). However, the retinol content coming from raw materials would be exactly the same in CONTROL and restricted diets (formulation is equal except for the vitamin-mineral premix) and thus, can be disregarded.

Sample collection

Ten pigs per treatment were slaughtered at the end of the growing period at101.4 kg (SD = 4.1 kg) LW and the remaining pigs at 157.9 kg (SD = 7.0 kg) LW (Industrias Cárnicas Alonso, S.L., Alcaudete de la Jara, Toledo, Spain). In the slaughterhouse, carcass length from the posterior edge of the symphysis pubica to the anterior edge of the first rib, ham length from the anterior edge of the symphysis pubica to the articulatio tarsi, and ham circumference at its widest point were measured on the left side of each carcass. Backfat thickness at the 10th rib on the midline of the carcass (skin included) was also measured. Carcass, ham and foreleg weights were recorded after slaughter. Samples from liver, heart and subcutaneous adipose tissue at the level of the last rib were taken, weighed, vacuum-packed in low-oxygen permeable film and kept frozen at –20ºCuntil fatty acid composition could be measured. Backfat was separated into outer and inner layers, which were independently assayed for fatty acid composition. In addition, at 157.9 kg (SD = 7.0 kg) slaughter weight, a 5 centimetres thick transversal slice was cut from the widest region of each ham (at the stifle joint level) and muscle, bone and subcutaneous and intermuscular fat were dissected and weighed. *Semimembranosus* (SM*)* and *Biceps Femoris* (BF*)* muscles and ham adipose tissue samples were taken and manipulated as described above.

Samples were obtained from adipose and hepatic tissues for gene expression analyses, immediately frozen in liquid nitrogen and kept at-80°C until analysis.

Laboratory analysis

Tissue fatty acid composition analyses.

Lipid extracts from adipose tissue samples (inner and outer layer) were extracted by the procedure proposed by Bligh and Dyer (1959). Fat extracts were methylated in the presence of sulphuric acid and analysed by gas chromatography. Fatty acid methyl esters (FAMEs) were identified by gas chromatography as described by López-Bote et al. (1997) using a Hewlett Packard HP-6890 (Avondale, PA, USA) gas chromatograph equipped with a flame ionization detector and a capillary column (HP-Innowax, 30 m × 0.32 mm i.d. and 0.25 μm polyethylene glycol-film thickness). A temperature program of 170 to 245ºCwas used. The injector and detector were maintained at 250ºC. The carrier gas (helium) flow rate was 2 ml/min. Results were expressed as grams per 100 grams of detected FAMEs.

Liver and ham muscle fatty acids were quantified by the one-step procedure described by Sukhijaand Palmquist (1988) in lyophilized tissue samples. Pentadecanoic acid (C15:0) (Sigma, Alcobendas, Madrid, Spain) was used as internal standard. Previously methylated fatty acid samples were identified by gas chromatography as described above.

Enzyme activity assays.

Glucose-6-phosphate dehydrogenase (G6PD) and L-3-hydroxyacyl-CoA dehydrogenase (L3HOAD) activities were measured in adipose tissue and liver samples and in heart samples, respectively. Enzymatic activity assays were carried out at growing and finishing periods. G6PD activity was determined as previously described by Alvarez et al. (2000). Briefly, samples from liver and subcutaneous backfat were weighed and homogenized (Turrax Ultra T18basic IKAw; Labortechnik, Staufen, Germany) in 3 volumes of ice-cold buffer (0.2 MTris-HCl, 0.5 Msucrose, 2 mMEDTA, pH 7.4). Homogenates were centrifuged at 20,000 rpm at 4ºC for 40 min and the supernatant was used to determine enzyme activity (Bautista et al., 1988).

Crude heart extracts were used to determine the activity of L3HOAD. Mitochondria were isolated from thawed hearts and activity determinations were performed as previously described by Saggerson (1982). Mitochondrial pellets were resuspended in 0.3 mol sucrose containing 10 mmol TrisHCl (pH 7.4 at 0°C) and 1 mmol EGTA per L and sonicated to obtain a mitochondrial supernatant after centrifugation at 20,000 g for 40 min at 4°C. The activity of L3HOAD was measured according to the spectrophotometric method proposed by Bradshaw and Noyes (1975).

The specific enzyme activities were expressed in IU (defined as μmol substrate converted to product per min at 30ºC) per mg of soluble protein. Soluble protein content was measured according to the method of Bradford (1976), using BSA as standard (chemicals from Sigma, Alcobendas, Spain). All the enzyme assays were conducted in duplicate at 30ºC. Initial rates were measured in all assays.

Gene expression.

Gene expression analysis was performed in samples from CONTROL and ER animals at101.4 kg LW (n = 20). Nineteen genes were selected as candidate genes based on their roles in adipogenesis regulation, lipid synthesis and metabolism, retinol signalling and energy homeostasis.

RNA was extracted from 50-100 mg liver and adipose tissue (inner layer) frozen samples using the Ribopure kit according to the manufacturer´s instructions (Ambion, Austin, TX). The RNA was quantified using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA) and its quality was evaluated with an Agilent Bioanalyzer 2100 device (Agilent Technologies, Palo Alto, USA). The RNA Integrity Number (RIN) values ranged from 7.9 to 8.4 in liver and from 7.1 to 9.2 in backfat. First-strand cDNA was synthesized using 1 μg of total RNA as template and treated with Superscript II (Invitrogen, Life Technologies, Paisley, UK) and random hexamers in a total volume of 20 μl.

Primer pairs used for quantification were designed using Primer Select software (DNASTAR, Wisconsin, USA) from the available GENBANK and/or ENSEMBL pig sequences, covering different exons to ensure amplification of cDNA. Primer sequences and amplicon lengths are indicated in Table 2.

Table 2: Primer design for qPCR, gene details and PCR efficiencies (eff, %) in the 2 analysed tissues: adipose tissue (A) and liver (L)

Gene name	Gene symbol	GenBank Acc. number	Forward primer sequence	Reverse primer sequence	Size, bp	A eff, %	L eff %
Transcriptional control of adipogenesis							
CCAAT/enhancer binding protein beta	CEBPB	NM001199889	GTGGCGCCGGCAAAACTT	GAGGGGGCAGGAGGAGAGGCAGAG	203	96.85	97.15
Peroxisome proliferator-activated receptor G	PPARG	DQ437884	GGCGAGGGCGATCTTGACAG	GATGCGAATGGCCACCTCTTT	148	99.9	99.3
Retinoic acid receptor alpha	RARA	XM003131474	TCCGCCGAAGCATCCAGAAGAAC	ACCTCCGGCGTCAGCGTGTAGC	217	92.9	93.7
Retinoic acid receptor, gamma	RARG	XM005652562	CTTCTTCCGCCGCAGCATCCA	AGCGACCCTTCTTCCTTCACCTC	195	90	86.5
Retinoid X receptor G	RXRG	NM001130213	GGGGTTGGCTCCATCTTTGA	ACCTGCCCGGCTGTTCTG	223	95.6	99.4
Sterol regulatory binding transcription factor 1	SREBP-1c	AY307771	TTGCGCAAGGCCATCGACTACATC	GTCTACCACCTCCGGCTTCACACC	180	94.75	91.25
Fatty acids biosynthesis							
Acetyl-CoA carboxylase alpha	ACACA	NM001114269	CTGAGAGCTCGTTTGAAGGAATA	TTTACTAGGTGCAAGCCAGACAT	281	89.35	91.95
Fatty acid elongase 6	ELOVL6	XR305072	AGAACACGTAGCGACTCCGAAGAT	GACATGCCGACCGCCAAAGATAA	182	87.15	90.25
Fatty acid synthase	FASN	NM001099930	GCAGGCGCGTGATGGGAATGGTG	GCCCGAGCCCGAGTGGATGAGCA	206	85.25	85.5
Glucose-6-phosphate dehydrogenase	G6PD	XM003360515	AGGCCGTGTACACCAAGATGATGA	TTGTGCAGCAGCGGCGTGAAGA	220	91.2	85.4
Malic Enzyme	ME1	X93016	GCCGGCTTTATCCTCCTCT	TCAAGTTTGGTCTGTATTTTCTGG	223	84.5	82.6
Stearoyl-CoA desaturase 1	SCD1	JN613287	TCCCGACGTGGCTTTTTCTTCTC	CTTCACCCCAGCAATACCAG	205	88.95	91.35
Fatty acids metabolism							
Acyl-CoA oxidase 1, palmitoyl	ACOX1	NM001101028	TGGCGGGCACGGCTATTCT	TGGCTGGGCAGGTCATTCA	195	90.4	98.55
Acyl-CoA synthetase long-chain family member 4	ACSL4	NM001038694	CCCGGTTGGTCAAGGCTATGGATT	CTCTGGGGTTTGGCTTGTCGTGAA	170	96.35	94.9
Retinoid signaling							
Cellular retinoic acid binding protein 2	CRABP2	NM001164509	GTACCACGGAGATCAACTTCAA	TGCCGTCATGGTCAGGA	200	95.5	90.95
Fatty acid binding protein 5	FABP5	AY841270	TCACCATCAAAACGGAGAG	TGACGCATACCACCACTAAT	210	98.45	89.25
Energy homeostasis							
Insulin-like growth factor 1	IGF1	NM214256	CATCCTCTTCGCATCTCTTCTACT	TGTGGCGCCCTCCGACTGCT	184	89.5	90.05
Insulin receptor	INSR	XM005654749	GGCCCTGTGACCCATGAAATCTT	TGGCCCGAACTCGAACGCTGTAAT	220	92.1	94.25
Leptin	LEP	GQ268935	GGCCCTATCTGTCCTACGTTGAAG	TGGAAGGCAGACTGGTGAGGAT	237	94.55	-

Standard PCRs on cDNA were carried out to verify amplicon sizes. Transcript quantification was performed using SYBR Green mix (Roche, Basel, Switzerland) in a LightCycler480 (Roche, Basel, Switzerland) in 384-well reaction plates. The qPCR reactions were prepared in a total volume of 20 µl containing 2.5 µl of cDNA (1/20 dilution), 10 µl of SYBR Green mix and 0.15 µM of both forward and reverse primers. As negative controls, mixes without cDNA were used. Cycling conditions were 95ºC for 10 min, followed by 45 cycles of 95ºC (15 s) and 60ºC (1 min) when the fluorescence was acquired. Finally, a dissociation curve to test PCR specificity was generated by one cycle at 95ºC (15s) followed by 60ºC (20s) and ramp up to 95°C with acquired fluorescence during the ramp to 0.01°C/s. Data were analysed with LyghtCycler480 SW1.5 software (Roche, Basel, Switzerland). All points and samples were run in triplets as technical replicates and dissociation curves were carried out for each individual replicate. Single peaks in the dissociation curves confirmed the specific amplification of the genes. PCR efficiency was estimated by standard curve calculation using 4 points of 5-fold cDNA dilutions from a pool of samples. Values of PCR efficiency are indicated in Table 2. C_p values were employed for the statistical analyses of differential expression. Four commonly used housekeeping genes were tested with geNorm software (Vandesompele et al., 2002) to evaluate their stability. *GAPDH* and *ACTB* were selected to normalize liver samples whereas adipose tissue normalization was carried out using *GAPDH* and *TBP* as housekeeping genes.

Statistical analysis

Phenotypic data were analysed as a completely randomized design using the general linear model (GLM) procedure in SAS version 9.2 (SAS Inst. Inc., Cary, NC; 2009). The mean and diet treatment were considered as systematic effects, and residual effects as random. The animal was the experimental unit for all analyses. Turkey'*s* test was used to identify differences between treatment means.

Time x dietary treatment and fat location x dietary treatment interactions were tested for the evolution of fatty acids classes (SFA, MUFA and PUFA) over time and for different fat locations (inner and outer layers of backfat and subcutaneous fat), respectively.

Statistical analysis of gene expression data was carried out following the method proposed by Steibel et al. (2009), which consists of the analysis of cycles to threshold values (C_p), for the target and endogenous genes using a linear mixed model. The following model was used for analysing the joint expression of the target and control genes in different tissues:

$$y_{gijkr} = TG_{gi} + L_{gj} + B_{gjk} + D_{ijk} + e_{gijkr}$$

where $y_{gijkr} = -log_2\left(E_g^{-Cp_{gijkr}}\right)$ $y_{gijk} = log_2\left(E_{gijk}^{-C_{gijk}}\right)$, *Eg* is the efficiency of the PCR of *g*th gene, C_{pgijkr} is the value obtained from the thermocycler software for the *g*th gene from the *r*th

replicate in a sample collected from the *k*th animal of the *j*th litter fed with the *i*th dietary treatment, TG_{gi} is the specific effect of the *i*th dietary treatment on the expression of gene *g*th, L_{gj} and B_{gjk} are specific random effects of the *j*th full-sib family and the *k*th pig on the expression of gene *g*th, D_{ijk} is a random sample-specific effect common to all the genes, and e_{gijkr} is a residual effect.

To test differences in the expression rate of genes of interest ($diff_{TG}$) between classes normalized by the endogenous genes, different contrasts were performed between the respective estimates of *TG* levels. Significance of $diff_{TG}$ estimates was determined with the *t* statistic. To obtain fold change (FC) values from the estimated $diff_{TG}$ values, the following equation was applied: $FC = 2^{-diff_{TG}}$. $\mathbf{FC = 2^{-diff_{TG}}}$ Asymmetric 95% confidence intervals (CI) were calculated for each *FC* value by using the standard error (SE) of the estimated difference: 95% CI from $2^{[-(diff]_{TG}+1.96\ x\ SE)}$ $\mathbf{FC_{UP} = 2^{[-(diff]_{TG}-1.96\ x\ SE)}}$ $2^{(-\mathrm{diff_{TG}}+1.96\mathrm{xSE})}$.

P-values < 0.05 were considered statistically significant.

3.5.4- Results

Effect of diet on carcass traits

No effect of dietary vitamin A level was observed on carcass traits at growing (101.4 kg LW) or fattening (157.9 kg LW) phases. The weights of ham components were also not affected by dietary treatment. Table 3 shows carcass and ham properties at 157.9 kg LW for the 3 dietary groups.

Table 3: Carcass and ham characteristics according to diet vitamin A level at the finishingphase. Percentage of weight of each component within a transversal 5 cm thick slice from the widest region of each ham (at the stifle joint level) of pigs slaughtered at the end of the fattening phase (157.9 ± 7 kg LW).

	DIET (MEAN)				*P*
Trait	C[1]	ER[2]	LR[3]	SEM	Diet
Carcass trait					
Carcass weight, kg	122.75	124.41	116.51	1.70	0.1555
Carcass length, cm	81.30	80.55	81.55	0.33	0.4486
Ham weight, kg	14.60	13.79	14.07	0.27	0.4653
Ham length, cm	44.65	43.85	44.80	0.24	0.2285
Ham perimeter, cm	77.15	75.60	75.10	0.45	0.1759
Foreleg weight, kg	8.70	8.72	9.10	0.14	0.3979
Backfat thickness, mm	63.50	62.50	60.70	1.41	0.7163
Ham component					
Biceps Femoris muscle, %	16.52	16.14	17.01	0.22	0.8501
Semimembranosus muscle, %	27.17	29.58	28.62	0.52	0.1899
Intermuscular fat, %	1.87	1.75	1.69	0.09	0.228
Subcutaneous fat, %	19.13	18.83	19.26	0.45	0.9292
Femur bone, %	3.58	3.72	3.87	0.11	0.3567
Remaining muscle, %	31.66	29.98	29.63	0.45	0.8194

Within a row, means without a common superscript differ ($P < 0.05$).
[1]C = Control = Control diet, 10000 UI vitamin A
[2]ER = Early restriction = Dietary vitamin A restricted diet from 2 months of age.
[3]LR = Late restriction = Dietary vitamin A restricted diet from 4 months of age.

Effect of diet on IMF content and fatty acid profile of backfat, liver and muscle samples

Fatty acid composition was analysed at growing and finishing phases in subcutaneous backfat and liver samples. Adipose and muscle samples obtained from the ham were only analysed at the finishing phase and results on the effect of diet on fat content and composition are shown in Tables 4 (subcutaneous ham fat) and 5 (intramuscular ham fat).

Regarding backfat fatty acid composition at the growing (101 kg LW) phase, we detected little effect of the diet. When mean comparisons were made in the outer layer fatty acids, C16:1 n-9 and C16:1 n-7, concentrations were higher in the ER group than in the CONTROL group (0.27 ± 0.01 vs. 0.34 ± 0.02, $P = 0.0006$ and 2.09 ± 0.04 vs. 2.36 ± 0.12, $P = 0.036$, respectively) but C20:1 and C20:2 contents were lower in the ER than the CONTROL group (1.73 ± 0.05 vs. 1.55 ± 0.05, $P = 0.027$ and 0.76 ± 0.02 vs. 0.68 ± 0.02, $P = 0.021$, respectively). In the inner layer, we did not detect any treatment effect on fatty acids.

In contrast, at the finishing (157 kg LW) phase, dietary treatment strongly affected fatty acid composition in both outer and inner layers of both backfat and ham subcutaneous fat. No

differences in diet effects were observed between fat layers. Results obtained for the two locations were similar, except for ∑n-3 fatty acids and n-6/n-3 ratio. A greater n-3 fatty acid content was observed in LR than in ER group in backfat while the opposite effect was observed in ham subcutaneous fat (P = 0.0236 and P = 0.0397, respectively, for the interaction between treatment and location). Table 4 shows the fatty acids profile in the outer layer of ham subcutaneous fat.

Table 4: Fatty acid composition (%) of subcutaneous fat (outer layer) from the ham at the end of the fattening period (157.9 ± 7 kg LW)

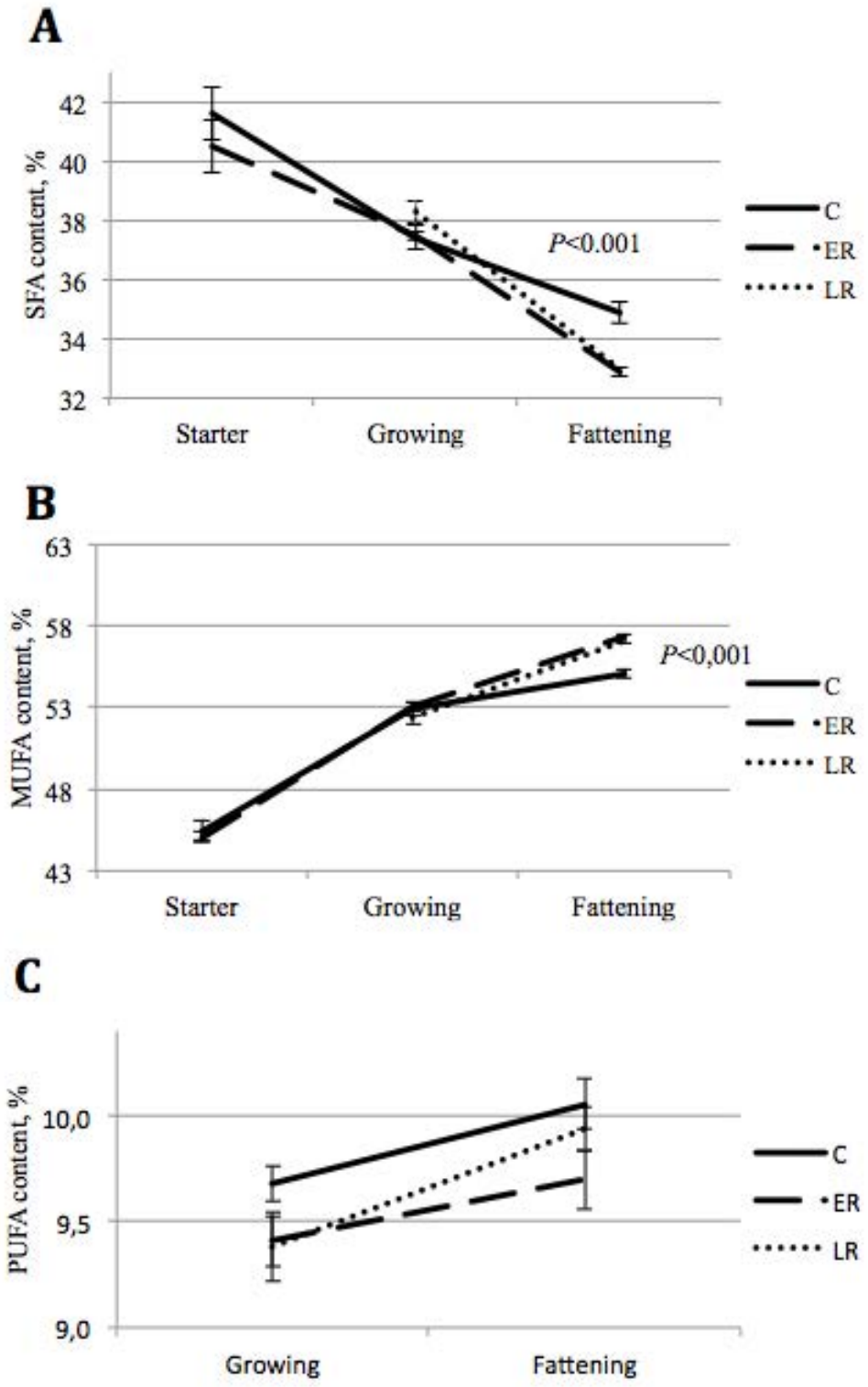
	DIET (MEAN)							*P*
Fatty acid	C[1]		ER[2]		LR[3]		SEM	Diet
C10:0	0.03	a	0.03	a	0.03	b	0.00	0.0028
C12:0	0.05	a	0.05	a	0.04	b	0.00	0.0008
C14:0	1.11	a	1.06	a	0.98	b	0.01	0.0003
C15:0	0.04		0.05		0.05		0.00	0.1102
C16:0	20.48	a	19.80	b	19.01	c	0.11	<.0001
C16:1 n9	0.27	b	0.29	ab	0.31	a	0.03	0.0283
C16:1 n7	1.66	ab	1.76	a	1.56	b	0.01	0.0395
C17:0	0.33		0.36		0.37		0.01	0.0674
C17:1	0.34	b	0.41	a	0.40	a	0.01	0.0027
C18:0	10.95	a	9.72	b	10.05	b	0.12	0.0009
C18:1	53.17	b	55.21	a	55.58	a	0.18	<.0001
C18:2 n6	7.99		7.79		7.78		0.06	0.2982
C18:3 n3	0.41	b	0.46	a	0.43	ab	0.01	0.0062
C20:0	0.23	a	0.19	b	0.23	a	0.01	0.0222
C20:1 n9	1.85		1.78		1.98		0.04	0.1019
C20:2 n6	0.75	ab	0.72	b	0.81	a	0.01	0.0278
C20:3 n6	0.18		0.18		0.21		0.01	0.1527
C20:4 n6	0.15	b	0.15	b	0.19	a	0.01	0.0426
ΣSFA	33.22	a	31.26	b	30.75	b	0.20	<.0001
ΣMUFA	57.29	b	59.44	a	59.83	a	0.21	<.0001
ΣPUFA	9.49		9.30		9.42		0.08	0.6213
UI[4]	77.16	b	78.99	a	79.68	a	0.22	0.0003
Σn3[5]	0.41	b	0.46	a	0.43	ab	0.01	0.0062
Σn6[6]	9.08		8.84		8.99		0.08	0.4471
Σn6/Σn3	22.33	a	19.43	b	21.05	a	0.27	0.0006
C16:1/C16:0	0.09	b	0.10	a	0.10	b	0.00	0.0006
C18:1/C18:0	4.88	b	5.70	a	5.56	a	0.06	0.0004
ΣMUFA/ΣSFA	1.73	b	1.90	a	1.95	a	0.01	<.0001

Within a row, means without a common superscript differ (P < 0.05).
[1]C = Control = Control diet, 10000 UI vitamin A
[2]ER = Early restriction = Dietary vitamin A restricted diet from 2 months of age.
[3]LR = Late restriction = Dietary vitamin A restricted diet from 4 months of age.
[4]UI = Unsaturation index = 1 × (% monoenoics) +2 × (% dienoics) +3 × (% trienoics) +4 × (% tetraenoics) +5 × (% pentaenoics) +6 × (% hexaenoics) (Hulbert et al., 2007)
[5]Σn3 = Sum of n-3 fatty acids
[6]Σn6 = Sum of n-6 fatty acids

Dietary vitamin A restriction produced an increase of MUFA and a decrease of SFA when compared to CONTROL diet at the end of the finishing period (Table 4). This effect was mostly due to an increase in oleic acid (C18:1) and a decrease in palmitic (C16:0) and stearic (C18:0) acids in samples from restricted groups. Conversely, the MUFA/SFA ratio was higher in both restricted groups. The increase in MUFA led to an increase in the unsaturation index despite the lack of effect on PUFA. CONTROL and LR groups showed higher values than ER group for the n-6/n-3 ratio.

Also noticeable was an effect of time over the fattening period on SFA, MUFA and PUFA content in backfat regardless the effect of diet (Fig. 1).Furthermore, dietary vitamin A restriction significantly affected SFA and MUFA evolution (Fig. 1-A and 1-B).The ER and LR groups showed a higher slope in the trend line than the CONTROL group and a time x dietary treatment interaction was observed ($P < 0.001$, for SFA and MUFA), whilst, PUFA evolution was not affected by dietary treatment (Fig. 1-C).

Figure 1: Evolution of treatment effects on: SFA (A); MUFA (B) and PUFA (C) at the end of the growing phase (Growing) and fattening phase (Fattening) in backfat. P-values correspond to the interaction between treatment and time



Fatty acid composition of ham muscles (BF and SM) at the finishing phase is shown in Table 5. In agreement with subcutaneous fat findings, MUFA content was higher in vitamin A restricted animals and this effect was more evident when the restriction was longer. The opposite effect was observed for SFA, although in this case the effect was not affected by the treatment duration. The increase in MUFA was mainly due to an increase in C18:1 at expense of C18:0 and C16:0fattyacids, as can be observed in the MUFA/PUFA and C18:1/C18:0 ratios. This response was of greater magnitude in ham muscles than in subcutaneous fat. Besides, PUFA content was lower in the ER group than in the CONTROL group while the LR group showed intermediate values (Table 5).

Table 5: Fatty acid composition (%) of main ham muscles at the end of the fattening phase (157.9 ± 7 kg LW)

	Biceps Femoris muscle					*Semimembranosus muscle*				
	DIET (MEAN)				*P*	DIET (MEAN)				*P*
	C[1]	ER[2]	LR[3]	SEM	Diet	C	ER	LR	SEM	Diet
IMF[4], %	7.74	8.76	7.38	0.48	0.3687	4.60 b	6.78 a	5.59 ab	0.26	0.02
C14:1	1.30 a	1.18 b	1.16 b	0.02	0.0019	1.29 a	1.17 b	1.14 b	0.01	0.0008
C15:1	0.78	0.65	0.82	0.04	0.1753	1.22	0.93	1.12	0.05	0.0719
C16:0	22.55 a	21.53 b	21.44 b	0.11	0.0006	21.58 a	20.84 b	20.68 b	0.11	0.008
C16:1 n9	0.28	0.26	0.30	0.05	0.6688	0.25	0.22	0.27	0.06	0.2711
C16:1 n7	3.57 a	3.62 a	3.28 b	0.02	0.0143	3.28	3.31	3.09	0.02	0.5206
C17:0	0.28	0.26	0.30	0.01	0.0954	0.37	0.33	0.38	0.01	0.0894
C17:1	0.23 b	0.25 a	0.27 a	0.00	0.0009	0.25 c	0.27 b	0.30 a	0.00	<.0001
C18:0	9.52 a	8.53 c	9.02 b	0.07	<.0001	9.38 a	8.49 c	8.90 b	0.06	<.0001
C18:1 n9	46.81 c	49.78 a	48.43 b	0.07	0.0004	45.04 b	48.67 a	47.23 a	0.08	0.0005
C18:1 n7	3.85	3.95	3.80	0.26	0.6919	4.21	4.14	4.09	0.33	0.8198
C18:2 n6	6.64	6.14	6.85	0.15	0.1376	7.91	7.07	7.70	0.15	0.0644
C18:3 n3	0.30 c	0.33 b	0.35 a	0.00	0.0007	0.34 b	0.36 b	0.40 a	0.01	0.0006
C18:4 n3	0.13 b	0.14 ab	0.15 a	0.00	0.049	0.17	0.17	0.18	0.00	0.462
C20:0	0.13	0.12	0.13	0.00	0.2749	0.13	0.12	0.13	0.00	0.2592
C20:1 n9	0.98	1.00	0.96	0.01	0.4902	1.06	1.12	1.08	0.02	0.4052
C20:2 n6	0.32 ab	0.30 b	0.34 a	0.01	0.0289	0.38 a	0.35 b	0.39 a	0.00	0.0154
C20:3 n6	0.18	0.15	0.18	0.01	0.193	0.22 a	0.17 b	0.21 a	0.01	0.0175
C20:4 n6	1.63	1.33	1.67	0.07	0.1512	2.30 a	1.73 b	2.09 ab	0.09	0.036
C20:5 n3	0.11 b	0.11 ab	0.12 a	0.00	0.0274	0.13	0.12	0.13	0.00	0.0832
C22:4 n6	0.18	0.14	0.18	0.01	0.0794	0.22 a	0.17 b	0.20 ab	0.01	0.0222
C22:5 n3	0.17 ab	0.16 b	0.20 a	0.01	0.0292	0.22 ab	0.19 b	0.23 a	0.01	0.0442
C22:6 n3	0.08	0.06	0.07	0.00	0.2168	0.10	0.08	0.10	0.00	0.2697
ΣSFA	32.48 a	30.44 b	30.88 b	0.14	<.0001	31.46 a	29.79 b	30.10 b	0.14	0.0001
ΣMUFA	57.81 c	60.69 a	59.02 b	0.21	<.0001	56.60 c	59.83 a	58.32 b	0.26	0.0001
ΣPUFA	9.71	8.87	10.10	0.24	0.1181	11.93 a	10.38 b	11.58 ab	0.24	0.0374
UI[5]	82.11	82.63	84.37	0.51	0.1866	87.07	85.75	87.71	0.49	0.2655
Σn3[6]	0.77 b	0.80 b	0.88 a	0.01	0.0055	0.90 b	0.89 b	1.00 a	0.01	0.0077
Σn6[7]	8.94	8.07	9.22	0.23	0.118	11.03 a	9.49 b	10.58 ab	0.23	0.0346
Σn6/Σn3	11.57 a	10.09 b	10.44 b	0.16	0.0017	12.23 a	10.64 b	10.58 b	0.19	0.002
C16:1/C16:0	0.17	0.18	0.17	0.00	0.0539	0.16	0.17	0.16	0.00	0.5353
C18:1/C18:0	5.33 c	6.31 a	5.80 b	0.02	<.0001	5.26 c	6.24 a	5.77 b	0.02	<.0001
ΣMUFA/ΣSFA	1.78 c	2.00 a	1.91 b	0.01	<.0001	1.80 b	2.01 a	1.94 a	0.01	<.0001

Within a row, means without a common superscript differ ($P < 0.05$); [1]C = Control = Control diet, 10000 UI vitamin A;[2]ER = Early restriction = Dietary vitamin A restricted diet from 2 months of age; [3]LR = Late restriction = Dietary vitamin A restricted diet from 4 months of age.
[4]IMF = Intramuscular fat; [5]UI = Unsaturation index = 1 × (% monoenoics) +2 × (% dienoics) +3 × (% trienoics) +4 × (% tetraenoics) +5 × (% pentaenoics) +6 × (% hexaenoics) (Hulbert et al., 2007); [6]Σn3 = Sum of n-3 fatty acids; [7]Σn6 = Sum of n-6 fatty acids.

An increase was also observed in n-3 PUFA content in the LR group when compared to the ER and CONTROL groups in BF muscle and to a lesser extent in SM muscle. On the other hand n-6 PUFA content in SM muscle was lower in the ER than in the LR and the CONTROL groups but there was no effect on BF muscle. The effect of diet on n-6/n-3 ratio was similar in both muscles; the CONTROL group showing higher ratio than both restricted groups (Table 5).

Finally, in SM muscle, a higher IMF content was observed in the ER group ($P = 0.020$) than in the CONTROL and the LR groups. The lowest IMF content was observed in the CONTROL group, whereas the LR group showed an intermediate value, which was not different from that of any of the other groups.

Regarding liver fatty acids composition, there was a slight effect of dietary vitamin A at both periods. Specifically, at the growing phase, an effect on polyunsaturated n-3 fatty acids C18:4 (0.40 ± 0.07 vs. 0.47 ± 0.06, $P = 0.037$) and C22:5 (1.03 ± 0.23 vs. 1.40 ± 0.27, $P = 0.004$) was found, with higher content of both FA in the ER group than in the CONTROL group. The LR group showed intermediate values. Those differences were not detected at the finishing phase due to the lower magnitude of the effects (Table 6). However, we observed some treatment effects on fatty acid profile in liver at the finishing phase, which involved minor fatty acids, such as C15:0, C16:1 n-9, C17:1, C20:3 n-6 and C20:5 n-3.

Table 6: Fatty acid composition (%) of liver at the end of the fattening phase (157.9 ± 7 kg LW)

	DIET (MEAN± SD)				p
	C	ER	LR	SEM	Diet
C14:0	1.11	1.12	1.11	0.06	0.9909
C15:0	0.10 b	0.11 a	0.11 a	0.00	0.0123
C15:1	0.10	0.10	0.08	0.01	0.3972
C16:0	20.64	18.37	20.53	0.92	0.2342
C16:1 n9	1.20 b	1.38 a	1.39 a	0.05	0.0325
C16:1 n7	1.93	2.07	2.13	0.12	0.5831
C17:0	0.24	0.24	0.42	0.07	0.1757
C17:1	0.38 b	0.43 ab	0.52 a	0.02	0.0025
C18:0	15.59	14.91	12.43	0.86	0.0797
C18:1 n9	33.23	35.79	36.03	1.02	0.1704
C18:1 n7	1.65	1.73	1.76	0.05	0.3849
C18:2 n6	11.15	11.24	11.52	0.29	0.7104
C18:3 n3	0.45	0.47	0.46	0.02	0.8137
C18:4 n3	0.49	0.55	0.58	0.03	0.1053
C20:0	0.07	0.08	0.07	0.01	0.4341
C20:1 n9	0.47	0.46	0.48	0.02	0.8324
C20:2 n6	0.30	0.31	0.31	0.02	0.9596
C20:3 n6	0.19 b	0.30 a	0.23 ab	0.03	0.0485
C20:4 n6	8.20	7.86	7.43	0.43	0.5523
C20:5 n3	0.06 b	0.07 a	0.07 a	0.00	0.0308
C22:1 n9	0.17	0.17	0.18	0.01	0.5807
C22:4 n6	0.58	0.55	0.48	0.05	0.3804
C22:5 n3	0.80	0.80	0.87	0.06	0.6722
C22:6 n3	0.86	0.86	0.75	0.08	0.6714
ΣSFA	37.75	34.83	34.67	1.10	0.1508
ΣMUFA	39.17	42.16	42.61	1.09	0.1088
ΣPUFA	23.09	23.01	22.72	0.67	0.9389
UI	110.55	112.92	111.57	2.37	0.8100
Σn3	2.66	2.75	2.74	0.10	0.8109
Σn6	20.23	19.95	19.74	0.59	0.8702
Σn6/Σn3	7.65	7.31	7.22	0.19	0.3177
C16:1/C16:0	0.15	0.21	0.17	0.02	0.2613
C18:1/C18:0	2.29	2.62	3.54	0.40	0.1608
ΣMUFA/ΣSFA	1.05	1.24	1.25	0.07	0.1257

Within a row, means without a common superscript differ ($P < 0.05$).

[1]C = Control = Control diet, 10000 UI vitamin A

[2]ER = Early restriction = Dietary vitamin A restricted diet from 2 months of age.

[3]LR = Late restriction = Dietary vitamin A restricted diet from 4 months of age.

[4]UI = Unsaturation index = 1 × (% monoenoics) +2 × (% dienoics) +3 × (% trienoics) +4 × (% tetraenoics) +5 × (% pentaenoics) +6 × (% hexaenoics) (Hulbert et al., 2007)

[5]Σn3 = Sum of n-3 fatty acids

[6]Σn6 = Sum of n-6 fatty acids

Effect of diet on enzyme activity

G6PD activity in adipose or hepatic tissues was not significantly affected by the treatment. Nevertheless, in adipose tissue at the finishing phase a trend (P = 0.087) to higher activity was observed for the ER group when compared to the LR and CONTROL groups (0.021 ± 0.009 UI/mg protein, 0.015 ± 0.003 UI/mg protein and 0.015 ± 0.004 UI/mg protein, respectively).

L3HOAD activity was assayed in heart muscle samples. Dietary treatment produced no significant effect, but at finishing, a trend to higher activity was observed in vitamin A restricted groups (0.026 ± 0.008 and 0.028 ± 0.004 UI/mg protein for ER and LR, respectively) than in the CONTROL group (0.021 ± 0.005 UI/mg protein) (P = 0.067).

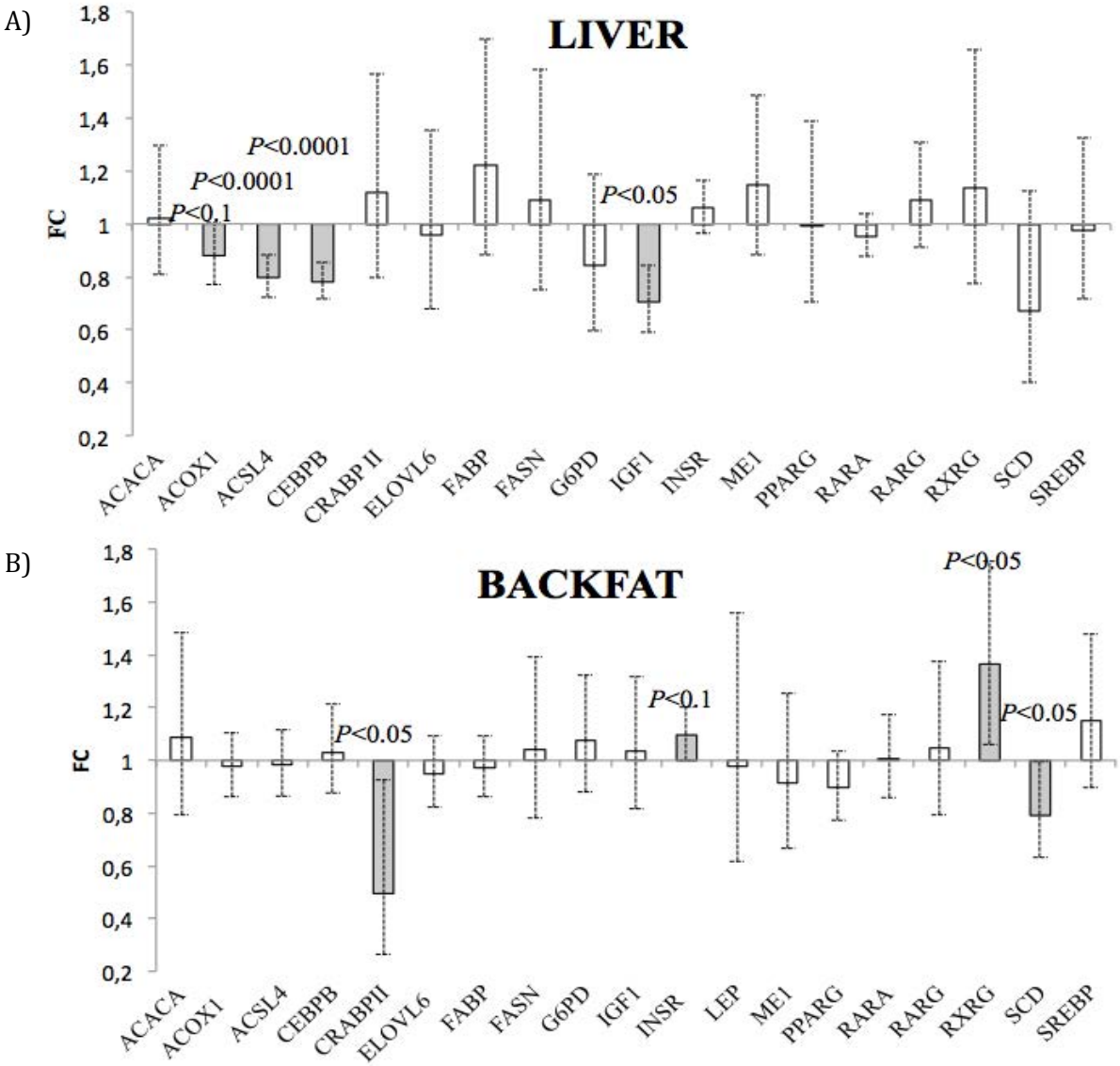
Effect of dietary vitamin A level on candidate gene expression

Gene expression was assessed in liver and adipose tissue samples of ER and CONTROL animals slaughtered at 100kg LW. The expression of 19 genes was assessed in adipose tissue but only 18 genes could be analysed in hepatic tissue due to lack of expression of the *Leptin* gene in this tissue.

Results obtained for liver are presented in Fig. 2A. Three out of the 18 candidate genes showed differential expression conditional on diet, and all of them were upregulated in the ER group: *ACOX1*, *CEBPB* and *IGF1* (P < 0.0001, P < 0.0001 and P = 0.0002 respectively). A difference between groups which did not reach statistical significance (P = 0.059) was observed for *ACSL4* gene expression, also with upregulation in ER group.

Regarding adipose tissue, results are shown in Fig. 2B. Three genes were differentially expressed between groups. The *CRABPII* and *SCD* genes were upregulated in the ER group (P = 0.028 and P = 0.045, respectively) whereas *RXRG* was upregulated in the CONTROL group (P = 0.016). Furthermore, *INSR* gene tended to show higher values in the CONTROL group (P = 0.052).

Figure 2: Change, expressed as Fold Change (FC) in candidate gene expression of control group with respect to the dietary vitamin A restricted group, in hepatic tissue (A-LIVER) and in carcass adipose tissue (B-BACKFAT) at the end of the growing phase (101.4 ± 4.1 kg LW)



3.5.5- Discussion

Vitamin A is a well-known regulatory factor involved in processes like immunity, vision, reproduction, growth and development (Blaner and Olson, 1994). The effect of vitamin A and its main active metabolite, retinoic acid (**RA**), on cell growth and differentiation and their importance in adipose tissue biology, obesity and type II diabetes have become apparent in recent years (Frey and Vogel, 2011). It was reported that RA regulates cell differentiation and proliferation, and prevents the process of adipogenic differentiation *in vitro* (Kuri-Harcuch, 1982; Castro-Muñozledo et al., 1987). Therefore it was hypothesized that vitamin A restriction would influence adipocyte

differentiation and hence marbling in beef cattle (Oka et al., 1998).Several studies assessed the effect of vitamin A status on productive traits and meat quality in different species including pig (D'Souza et al., 2003; Olivares et al., 2009a; Olivares et al., 2011), but diverse and even controversial results have been reported. Vitamin A is a lipophilic compound, and thus, dietary vitamin A restriction is expected to lead to a decrease in body vitamin A storage (mainly liver and fat). This was observed in our dietary vitamin A restricted pigs, as reported in a previous work (Ayuso et al., 2015c).

Effect of dietary vitamin A level on carcass characteristics and IMF content

Although vitamin A is essential for correct growth and development in mammals, its restriction did not produce any detrimental effect on weight or carcass length in our long-term experiment. Average daily weight gain, average daily feed intake and feed conversion ratio were also not affected by dietary vitamin A treatment, as previously reported (Ayuso et al., 2015c). These results are in agreement with other studies conducted in pigs (Olivares et al., 2011). Moreover, backfat thickness did not differ among treatments according to our results, as observed by Olivares et al. (2011).

In contrast to this lack of effect on subcutaneous fat, we observed a47% increase in IMF content (from 4.60 to 6.78%) in SM muscle as a consequence of vitamin A removal from the feed mix. Subcutaneous fat cells differentiate earlier than IMF adipose cells (Andrews, 1958; Hauser et al., 1997; Gondret and Lebret, 2002). It was reported that in the postnatal pig, subcutaneous fat accretion is mainly due to hypertrophy whereas only an early-stage-hyperplasia occurs in this depot. However, hyperplasia is the main event taking place in the IMF depot during the early post-natal period (Poulos et al., 2010). As vitamin A has a negative influence on adipocyte differentiation, tissues undergoing hyperplasic processes may be more sensitive to changing levels of dietary vitamin A.

However, no effect on IMF content was observed in BF muscle. This could be due to the greater amount of IMF deposited in BF in all the 3 experimental groups (from 7.38% in LR to 8.76% in ER group), which are among the highest values reported in Iberian swine muscle. For example, in *Masseter* muscle values range from 2.4% to 4.5% (Muriel et al., 2004), in *Longissimus Dorsi* from 3.7% (Muriel et al., 2004) to 6.3% (Ayuso et al., 2014) and in BF from 4.2% (Ventanas et al., 2006) to 7.1% (Fuentes et al., 2014). Thus, the very high levels of IMF observed in BF muscle might limit its responsiveness to the dietary intervention.

The IMF increase in one of the main ham muscles in response to dietary vitamin A restriction is a significant finding in the search for nutritional strategies for improving meat quality, particularly aimed to the production of high quality dry cured hams. Besides, this approach has important complementary advantages such as the lack of increase of backfat thickness and the reduction in feed costs resulting from suppression of vitamin A in the premix. Vitamin A, together with vitamin

E, are the most expensive vitamins used in swine feeds (31% and 2l% of total vitamin supplements cost, respectively) (Fraga and Villamide, 2000).The estimated cost reduction in vitamin A-restricted feed is 9 cents per ton of feed, which corresponds to 0.04% of total feed cost. Since vitamin A and E are both lipophilic vitamins, they compete for accumulation in cells and tissues and thus, restriction in vitamin A would favour vitamin E accumulation, which has also important benefits on meat stability and shelf-life.

Effect of diet on fatty acids profile

Dietary vitamin A withdrawal led to an increase in MUFA, a decrease in SFA and therefore an increase in desaturation indexes (MUFA/SFA, C16:1/C16:0, C18:1/C18:0) and in the unsaturation index. These effects are consistent in all analysed tissues (subcutaneous backfat, subcutaneous ham fat and intramuscular ham fat), except liver, which seems to be less sensitive to the restriction of vitamin A in relation to FA composition.

Previous studies reported an effect of genetic background on responsiveness of liver fatty acid composition to dietary vitamin A level. Lean pigs showed a noticeable effect of dietary vitamin A restriction on liver fatty acid profile (Olivares et al., 2011) whilst a slight effect was observed in genetically fat pigs (Olivares et al., 2009), in agreement with our results.

Previous studies assessed the effect of dietary vitamin A level in subcutaneous fat, with conflicting results. In general, it is considered that low doses or the complete withdrawal of dietary vitamin A in feeds lead to an increase in the desaturation index, as observed in swine (Olivares et al., 2009a; Olivares et al., 2011). Those results are in agreement with the ones presented here.

Limited information exists on the effect of vitamin A restriction on IMF fatty acid composition. Olivares et al. (2009a, 2011) observed no effect in genetically lean pigs. However, Olivares et al. (2009b) found similar results to those reported in the present study in genetically fat pigs (Duroc crossbred), with an increase in MUFA, and a concomitant decrease in SFA in the neutral lipids fraction and an increase in PUFA and decrease in SFA in polar lipids fraction of *Longissimus Dorsi* muscle lipids. It was then hypothesized that genetic background plays an important role on the effect of dietary vitamin A level on IMF and subcutaneous fat composition in pigs, which seems to be confirmed in our experiment, conducted in an extremely obese swine genotype.

Regarding the evolution of FA classes in backfat over the experimental period, the overall trend was a decrease in SFA concentration while MUFA and PUFA proportions increased. Changes in FA profile related to age have been previously described, but results are conflicting. These results are dependent on the age of sampling, breed, diet and tissue (it was reported that changes in FA profile of backfat overtime were smaller than in IMF (Bosch et al., 2012)). These authors observed an increase of MUFA to the detriment of PUFA while SFA remain unchanged in IMF and subcutaneous backfat in pigs. On the other hand, other authors found an increase in SFA and MUFA together with a decrease in PUFA (Wood et al., 2008; Lo Fiego et al., 2010; Raj et al., 2010). However, in a study

carried out in Torbiscal pigs slaughtered at similar ages to those in the present study (i.e. 8 and 12 months old) SFA decreased and MUFA and PUFA proportions increased in backfat when animals matured (Daza et al., 2007), which is consistent with our results. Besides the time effect, we observed an interaction between time and dietary vitamin A level in SFA and MUFA, but not in PUFA proportions, in agreement with the lack of effect on fatty acids profile observed at the growing phase and the noticeable effect observed at finishing on MUFA and SFA.

Effect of the dietary vitamin A level on candidate gene expression

Vitamin A, and specifically its metabolite, RA, is a well-known transcriptional regulator. Balmer and Blomhoff (2002) established that more than 500 genes are regulated by RA. Indeed, it has been established that vitamin A exerts its effects on adipose tissue via regulation of expression of several genes involved in adipogenesis and lipid metabolism (Schwarz et al., 1997; Bonet et al., 2003; Daniel et al., 2004). Thus, we hypothesized that a withdrawal of dietary vitamin A would affect the expression of genes involved in adipocyte differentiation and FA metabolism, which might lead to the phenotypic changes reported above.

A gene expression study was performed with samples obtained from ER and CONTROL pigs at the growing phase. This sampling was selected based on the results observed in phenotypic analysis, where the main differences occurred at the finishing phase and between CONTROL and ER groups. We assumed that phenotypic changes take place after changes in gene expression that drive them. Therefore, we hypothesized that gene expression changes should take place before phenotypic changes were observed. On the other hand, in order to study the effect of dietary vitamin A level on genes affecting adipocyte differentiation, a growing phase sampling is preferred, because adipocyte differentiation occurs mainly during growth and to a much lesser extent during adulthood.

In liver samples, 3 genes (*ACOX, CEBP, IGF1*) out of the 18 assessed were differentially expressed between groups and an additional one (*ACSL4*) was close to statistical significance. All of them were upregulated in the ER group. Within those genes, *ACOX1* and *ACSL4* showed 1.13-fold and 1.25-fold upregulation in ER group, respectively, and both are involved in fatty acid metabolism. *ACSL4* gene codes for an isozyme of the long-chain fatty-acid-coenzyme A ligase family, which converts free long-chain fatty acids into fatty acyl-CoA esters, and thereby play a key role in lipid biosynthesis and fatty acid degradation, mainly by β-oxidation (Soupene and Kuypers, 2008). In agreement with this, the G6PD enzyme, involved in FA biosynthesis, tended to be more activated in the ER group than in the LR and CONTROL groups.

On the other hand, the *ACOX1* gene codifies the first enzyme of the fatty acid beta-oxidation pathway, which catalyses the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs. These enzymes, involved in fatty acid metabolism, are induced during the late differentiation process of adipogenesis (Gregoire et al., 1998) and their upregulation requires the intervention of PPARG, considered as the master regulator of adipogenesis. Vitamin A negatively affects the adipocyte

differentiation process *in vitro*, likely via downregulation of PPARG expression (Bonet et al., 2003). We did not find PPARG expression changes between the experimental groups, however, because gene expression is a dynamic process, it is possible that we did not observe changes in PPARG due to the timing of sampling but we still could observe changes in expression in its downstream genes such as *ACOX1* and *ACSL4.* The downregulation of those genes in the CONTROL animals is consistent with the role of vitamin A as an inhibitor of adipogenesis.

Regarding *CEBPB* and *IGF1*, both of them play a role in the control of adipogenesis. *IGF1* expression is 42% higher in the ER group and is known to be involved in cell growth and differentiation processes (Sara and Hall, 1990) and adipogenesis (Wabitsch et al., 1995). Thus, an increase in its expression in restricted animals could lead to bigger and fatter animals. It has been reported that adipogenesis is not an exclusively early-in-life process and that adipocyte numbers increase under certain circumstances in growing and adult animals (Margareto et al., 2001; Faust et al., 1978). Hence an increase in adipocyte content could be expected in animals showing greater *IGF1* gene expression (i.e. ER group). This is in agreement with the phenotypic data observed in SM muscle.

The *CEBPB* gene is important in the regulation of genes involved in immune and inflammatory responses as well as in the signalling pathway for adipocyte differentiation. It has been widely accepted that *CEBPB* expression arises early in the adipocyte differentiation process (Yeh et al., 1995) and that this increase is responsible for the further activation of downstream genes, such as *CEBPα* and *PPARG* (Rosen et al., 2000). Moreover, antiadipogenic effects of RA have been shown to be exerted by blocking *CEBPB*-mediated induction of downstream genes (Schwarz et al., 1997), which could lead to a stop in the adipogenesis process. In the present study we observed a 28%increase in *CEBPB* expression in animals receiving no vitamin A in the feed as well as higher amount of IMF in SM muscle, thus suggesting that antiadipogenic effects of RA are driven not only by blocking CEBPB activity but also by downregulation of gene expression.

Regarding gene expression analysis in backfat, we found 3 genes differentially expressed between treatments (*CRABPII, RXRG and SCD*) as well as 1 gene (*INSR*) showing a trend.

SCD is the main enzyme involved in FA desaturation processes, which converts saturated fatty acids (mainly palmitic and stearic acid) into their corresponding Δ9-monounsaturated fatty acids (Smith et al., 1999; Paton and Ntambi, 2009). The *SCD* gene has an important effect on fatty acid profile and thus, on meat quality. We found an increase in *SCD* expression in restricted animals, suggesting a negative effect of vitamin A on *SCD* transcription. Conflicting results are found in different species, which suggest an influence of the genetic background in *SCD* expression response to dietary vitamin A. In the present study the change in *SCD* gene expression is consistent with the observed changes in FA composition in body tissues.

The *CRABPII* gene showed higher (2-fold) expression levels in the ER group. This gene encodes a member of the retinoic acid binding protein family. The protein is a cytosol-to-nuclear shuttling protein, which transports RA to the nucleus and regulates access of RA to the nuclear retinoic acid

receptors. Thus, it is involved in the retinoid-signalling pathway (Dong et al., 1999). The increased expression of this gene in the restricted group may be a compensatory mechanism due to the scarcity of circulating retinol and RA in those animals. On the other hand, *CRABPII* has been reported as a gene expressed only in preadipocytes, but not after differentiation, when its expression is inhibited by *CEBPα* (Berry et al., 2010). This stage-dependent expression pattern may suggest a greater amount of preadipocytes in the ER group, which could lead to a greater adipose mass in older animals; this is consistent with the higher IMF content found in SM muscle at the finishing phase.

We also found a slight (10%) increase in *INSR* expression in the CONTROL group. The *INSR* gene codes for the insulin receptor, which mediates the pleiotropic actions of insulin, although other molecules such as IGF1 and IGF2 may bind this receptor as well (Siddle, 2011). *INSR* gene expression is high in mature adipocyte, while it is not present in preadipocytes (Reed and Lane, 1980). This finding, consistent with the differential expression found for *CRABPII* gene, supports the hypothesis that the preadipocyte population is more numerous in the ER group while the adipocyte population may be slightly higher in the CONTROL group, which suggests higher adipogenic potential in the dietary vitamin A restricted group. On the other hand, it has been reported that *INSR* gene expression is regulated by different factors, such as nutrient status, cell growth stage and ligand abundance (Mamula et al., 1990). Since *IGF1* can bind *INSR* and plasma IGF1 regulates *IGF1-Receptor* expression (Hernandez-Sanchez et al., 1997), it might be possible that the increased expression of *IGF1* in ER group downregulates *INSR* expression in these animals.

Most of the biological effects of RAs involve the activation of ligand-dependent transcription factors of the nuclear hormone receptor superfamily, the retinoid receptors. Two types of these receptors are known: the retinoic acid receptors (RARs), which are responsive to both all-trans RA and 9-cis RA, and the rexinoid receptors (RXRs), which are responsive to the 9-cis RA isomer specifically (Bonet et al., 2003). There are 3 subtypes of RXRs, named RXRA, RXRβ and RXRG (Chambon, 1996). There is lack of information on vitamin A regulation of *RXRG*, maybe due to its tissue-specific expression pattern (Dolle et al., 1994). However, regulation of the other two subtypes of RXRs has been reported, although it is still controversial (Bonet et al., 1997; Kuwata et al., 2000). Our work provides new evidence indicating that vitamin A significantly increases the *RXRG* expression level (36% greater expression in CONTROL than ER group).

Overall, our results show the influence of a vitamin A-restricted diet on IMF content and on fatty acids profile, which may lead to a higher meat quality. The results on meat characteristics are, in addition, consistent with the changes in gene expression found in these animals.

Changes in gene expression reported in the present study provide a better understanding of the mechanisms involved in vitamin A regulation of fat depots. Also, it is important to highlight that differences observed in gene expression were slight, which suggests that the regulation of these genes is a subtle process that might lead to important biological changes, especially for those that

are transcription factors, affecting several downstream genes. Besides, for genes with low expression level, it may be difficult to find differences among groups due to technical limitations.
On the other hand, due to vitamin A properties and metabolism, phenotypic and transcriptomic changes conditional on vitamin A restriction are expected to be less marked than in supplementation studies, because vitamin A is a lipophilic compound which is stored in animal tissues, mainly liver and fat. These storage depots are responsible for vitamin A homeostasis when the intake is limited, thus requiring a certain depletion time prior to observing privation effects. Besides, ingredients in feedstuff may contain low levels of vitamin A that may affect the repercussion of dietary vitamin A restriction on phenotype and gene expression.

3.5.6- Implications

Long term dietary vitamin A restriction in Iberian pigs led to an increase in IMF content in SM muscle, which would have a positive impact on cured ham quality, the highest value product from Iberian-type pigs. The positive effect of dietary vitamin A restriction is not attributed only to the increase in IMF content, but also to changes observed in the fatty acid profile in all adipose tissue depots assessed. Lower SFA content means healthier and softer fat, which is highly appreciated by Iberian ham consumers. The decrease in SFA is associated with an increase in MUFA but not PUFA concentration, which is important for meat shelf-life, because PUFA content is associated with fat rancidity.
The treatment also produced some changes in fatty acid profiles with implications beyond meat quality. The increase in n-3 PUFA, mainly C18:3 n3 (alpha-linoleic acid) led to a decrease in the n-6/n-3 ratio, important for consumer health. Lower n-6/n-3 ratios, like the ones observed in restricted animals are associated with better health and lower risk for heart disease. Moreover, effects on meat quality parameters are not accompanied by changes in backfat thickness or other carcass traits.
Most diet effects are similar in both restricted groups, but effects on IMF, MUFA content and backfat n-6/n-3 ratio are influenced by the timing of the treatment, with the effect on IMF being significant only for the ER treatment. Thus, long treatments would be required for the practical application of this nutritional strategy. These results open new improvement possibilities with an important application, especially in autochthonous and fatty swine breeds, in which IMF content and other meat quality parameters are a main issue.

4.-DISCUSIÓN GENERAL

4.1- EFECTO DEL TIPO GENÉTICO SOBRE EL TRANSCRIPTOMA Y ASPECTOS FENOTÍPICOS EN CERDOS IBÉRICOS PUROS Y CRUZADOS.

La mayor parte de la producción porcina actual corresponde a carne procedente de razas altamente seleccionadas, con un gran potencial de crecimiento y acumulación de proteína, que producen carnes muy magras. Sin embargo, en algunos países de la franja mediterránea, como España, la producción de este tipo de carne coexiste con la de razas locales no seleccionadas. Entre éstas destaca la producción de carne de cerdo ibérico, con mayor contenido en grasa y de más calidad, consumida en fresco o como producto curado. Estos productos se obtienen tanto de cerdos ibéricos puros como de cruzados con Duroc, en los que se incrementa la eficiencia de crecimiento y acumulación de proteína, a pesar de que se ha observado una disminución de la calidad de la carne (Ventanas et al., 2006). La disminución de la calidad se atribuye a un menor contenido en GIM y ácido oleico, probablemente asociado con diferencias en el metabolismo lipídico y energético entre ambos genotipos, que se han podido evidenciar incluso desde los primeros estadios del desarrollo (Óvilo et al., 2014). Sin embargo, es necesario seguir profundizando en el estudio de los mecanismos metabólicos y moleculares responsables de estas diferencias, así como descubrir la forma en que estos mecanismos podrían verse afectados por el estadio de desarrollo o por condiciones externas al animal, tales como la alimentación. En el presente trabajo se ha utilizado tecnología de secuenciación masiva RNA-Seq para investigar este aspecto en cerdos ibéricos puros y cruzados al nacimiento y a los cuatro meses de edad, considerados momentos críticos al tratarse del inicio del desarrollo postnatal y de un punto intermedio en la fase juvenil del crecimiento de estos animales, y que cubren el periodo de mayor diferenciación de adipocitos intramusculares (Hauser et al., 1997). El diseño experimental empleado ha permitido evaluar el efecto de la edad, el genotipo y el músculo sobre aspectos fenotípicos así como sobre el transcriptoma de los músculos BF y LD.

4.1.1- Aspectos fenotípicos

La edad modificó obviamente los parámetros de crecimiento, pero también el contenido y la composición de la GIM, mostrando los animales de 4 meses de edad un mayor contenido en GIM y en AGMI que los neonatos (Capítulo 2, tabla 2). Por otro lado, uno de los resultados fenotípicos más llamativos observados en el presente estudio es que los cerdos cruzados fueron más grandes y pesados al nacimiento, mientras que estas diferencias desaparecieron a los cuatro meses (Capítulo 2, tabla 2). La ventaja en el desarrollo de los animales cruzados observada al nacimiento es coherente con las diferencias de crecimiento prenatal descritas entre genotipos de porcino (Torres-Rovira et al., 2013). Por otra parte, una posible hipótesis para explicar la ausencia de diferencia

entre ambos genotipos al destete (Óvilo et al., 2014) y a los 4 meses de edad podría ser el desarrollo de un crecimiento compensatorio de los cerdos ibéricos durante la lactación y la fase inicial del crecimiento. Este crecimiento compensatorio podría verse favorecido por el "fenotipo ahorrador", característico de la raza ibérica, que facilitaría un crecimiento rápido en condiciones de disponibilidad de alimento. También se ha observado un fenómeno similar en relación a los niveles de colesterol y triglicéridos plasmáticos, que fueron significativamente mayores en ibéricos puros recién nacidos pero se igualaron a los 4 meses. Estos resultados indican que el metabolismo lipídico difiere entre razas porcinas y entre distintos estadios de desarrollo. Tales diferencias pueden deberse a una mayor capacidad de síntesis de lípidos en los fetos ibéricos, o a un menor consumo prenatal de los lípidos procedentes de la circulación materna, debido posiblemente al menor crecimiento que exigiría un gasto energético más reducido. Uno de los aspectos fenotípicos de mayor interés en este estudio es el contenido en GIM, que se ha estudiado en dos músculos, BF (en lechones al nacimiento) y LD (al nacimiento y a los 4 meses de edad). Resulta interesante observar que ya desde el nacimiento, los cerdos ibéricos puros mostraron un mayor contenido en GIM en el músculo BF (Capítulo 1, tabla 1) pero no en LD (Figura 22). Sin embargo, este mismo efecto del genotipo es apreciable en el músculo LD a los 4 meses de edad (Capítulo 2, tabla 2), probablemente debido a diferencias en el metabolismo y la capacidad de acumulación de grasa entre ambos músculos (Leseigneur-Meynier y Gandemer, 1991; Kim et al., 2008) a lo largo del desarrollo. Estas diferencias podrían también afectar a la composición de la GIM, ya que en el músculo BF se observaron efectos del genotipo en animales recién nacidos en cuanto al contenido en AGS (menor en IB) que no se evidenciaron en el músculo LD de lechones al nacimiento o a los 4 meses.

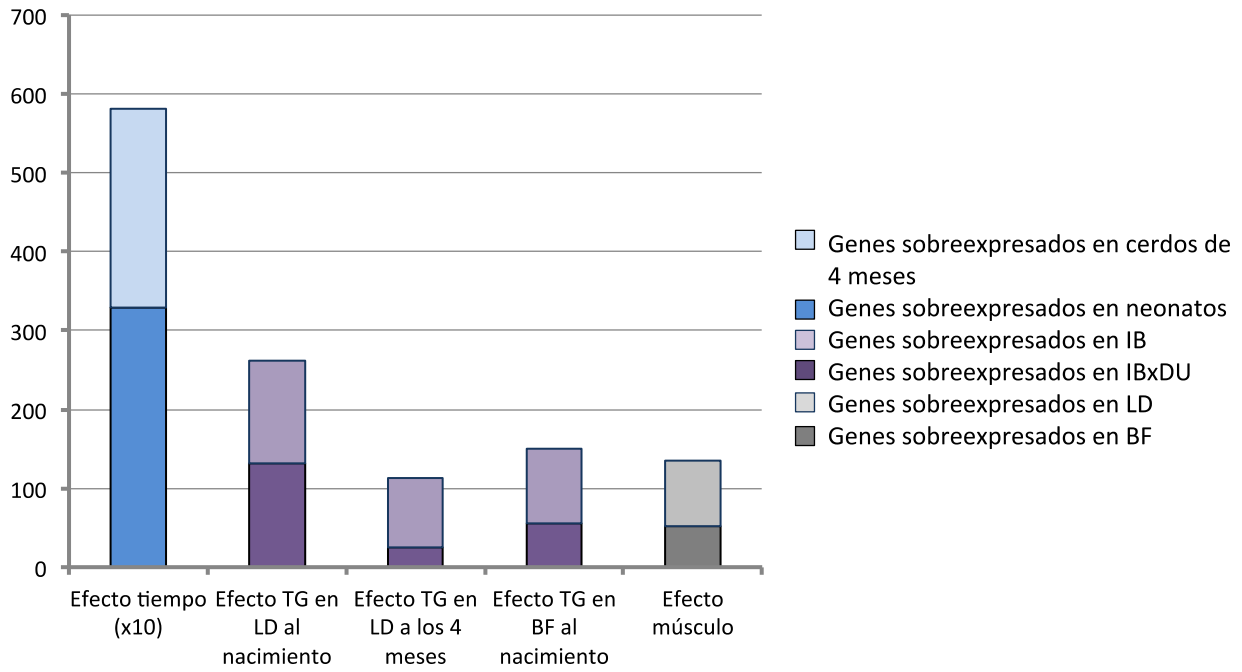
Figura 22 : Contenido en grasa intramuscular en los músculos *Longissimus dorsi* (LD) y *Biceps femoris* (BF) de cerdos ibéricos puros (IB) y cruzados con Duroc (IBxDU) al nacimiento.

LD: músculo *Longissimus dorsi*; BF: músculo *Biceps femoris*; IBxDU: cerdos ibéricos cruzados con Duroc al 50%; IB: cerdos ibéricos puros. Las letras *a* y *b* indican diferencia estadísticamente significativa (p = 0.014).

4.1.2- Efectos sobre la expresión génica

En el análisis del transcriptoma, la edad produjo el mayor efecto sobre la expresión génica con casi 5,800 genes DE entre el nacimiento y los 4 meses de edad. Los genes sobreexpresados en neonatos están involucrados en rutas metabólicas relacionadas con la biosíntesis de compuestos o con la proliferación celular, características de un estadio temprano y muy activo de crecimiento (Capitulo 2, tabla suplementaria 2). Sin embargo, en cerdos de 4 meses de edad, las rutas enriquecidas están relacionadas con procesos catabólicos orientados a obtener ATP, más propios de fases de crecimiento de menor intensidad (Vander Heiden et al., 2009). El tipo genético y el músculo modificaron la expresión de un número de genes considerablemente menor (Figura 23).

Figura 23: Número de genes diferencialmente expresados clasificados por efecto (tiempo, tipo genético y músculo) y por grupo dentro de cada efecto.



LD: músculo *Longissimus dorsi*; BF: músculo *Biceps femoris*; TG: Tipo genético: cerdos ibéricos cruzados con Duroc al 50% (IBxDU) y cerdos ibéricos puros (IB).

El efecto del músculo refleja un enriquecimiento en funciones y rutas metabólicas relacionadas con el metabolismo lipídico en el músculo BF cuando se comparó con el LD (Capítulo 2, tabla suplementaria 8), lo cual podría condicionar la mayor respuesta del BF a la inclusión de genética Duroc: mientras el músculo BF presentó diferencias en GIM desde el nacimiento éstas no fueron evidentes en el LD hasta los 4 meses de edad. Además, el efecto del genotipo parece estar también afectado por la edad y el tejido, puesto que los genes DE entre ibéricos puros y cruzados variaron con el músculo y fase de desarrollo en la que se encontraban (Capítulo 1 tabla suplementaria 2; capítulo 2, tabla suplementaria 4). Estos resultados sugieren la existencia de interacciones edad x tipo genético y músculo x tipo genético que no pudieron evaluarse en esta Tesis por limitaciones del software empleado, pero que deben ser consideradas en análisis futuros. En cualquier caso, se han identificado rutas metabólicas y funciones afectadas por el tipo genético comunes en ambos músculos y edades. Por ejemplo, la activación de rutas y funciones relacionadas con el metabolismo lipídico y glucídico en los animales ibéricos puros se observó tanto en el músculo BF como en LD a ambas edades, en concordancia con los resultados publicados por Óvilo y colaboradores (2014) en lechones al destete. Estos resultados, junto con la activación en el presente estudio de la ruta de señalización Wnt/Ca+, involucrada en el control de la homeostasis energética, sugieren un metabolismo energético más activo en los animales puros, lo que parece corroborar su mayor tendencia adipogénica. Además, el enriquecimiento de la ruta de señalización del receptor de GABA

(asociado con una alta tasa de metabolismo basal en humanos, (Wu et al., 2011)) en los cerdos cruzados de 4 meses de edad es un nuevo hallazgo que apoya estos resultados. Por otra parte, en el músculo BF, las rutas relacionadas con el metabolismo del colesterol se encontraron activadas en ambos genotipos (Capítulo 1 tabla suplementaria 4), lo que podría estar relacionado con el enriquecimiento en funciones y rutas metabólicas observado en el músculo BF cuando se comparó con el LD (Capítulo 2, tabla suplementaria 8), y con la diferente respuesta de cada músculo a la inclusión de genética Duroc: mientras el músculo BF presentó diferencias en GIM desde el nacimiento éstas no fueron evidentes en el músculo LD hasta los 4 meses de edad. Por otro lado, también se observó en ambos músculos un aumento de funciones y rutas metabólicas relacionadas con el crecimiento celular, y específicamente de las células musculares en animales cruzados al nacimiento. A pesar de que la mayoría de los genes involucrados fueron distintos en ambos tejidos, con predominancia de quimioquinas (*CCL19, CXCL13*) en el músculo BF (Capítulo 1, tabla suplementaria 2) y de colágenos y factores de crecimiento (*COL9A1* y *2*, *GDF5*) en el LD (Capítulo 2, tabla suplementaria 4), se identificó un gen DE en ambos músculos, así como en el LD de lechones al destete (Óvilo et al., 2014). Este gen es *MYH10*, una miosina no muscular que regula la remodelación del citoesqueleto de actina, necesaria durante el proceso de miogénesis (Vicente-Manzanares et al., 2009). El hecho de encontrarse DE de forma tan consistente en edades tempranas, lo convierte en un potente gen candidato relacionado con las diferencias en el desarrollo prenatal observadas entre ambos tipos genéticos. Sin embargo, a los 4 meses de edad, los animales puros mostraron un enriquecimiento de rutas relacionadas con el metabolismo de aminoácidos no esenciales (como serina, alanina o glicina) necesarios para la síntesis proteica y la proliferación celular (Capítulo 2, tabla 5), lo que hace pensar en una activación de los procesos relacionados con el crecimiento muscular en ibéricos puros en edad juvenil, de acuerdo con la hipótesis previamente planteada sobre el crecimiento compensatorio experimentado por estos animales durante la fase de crecimiento postnatal temprano.

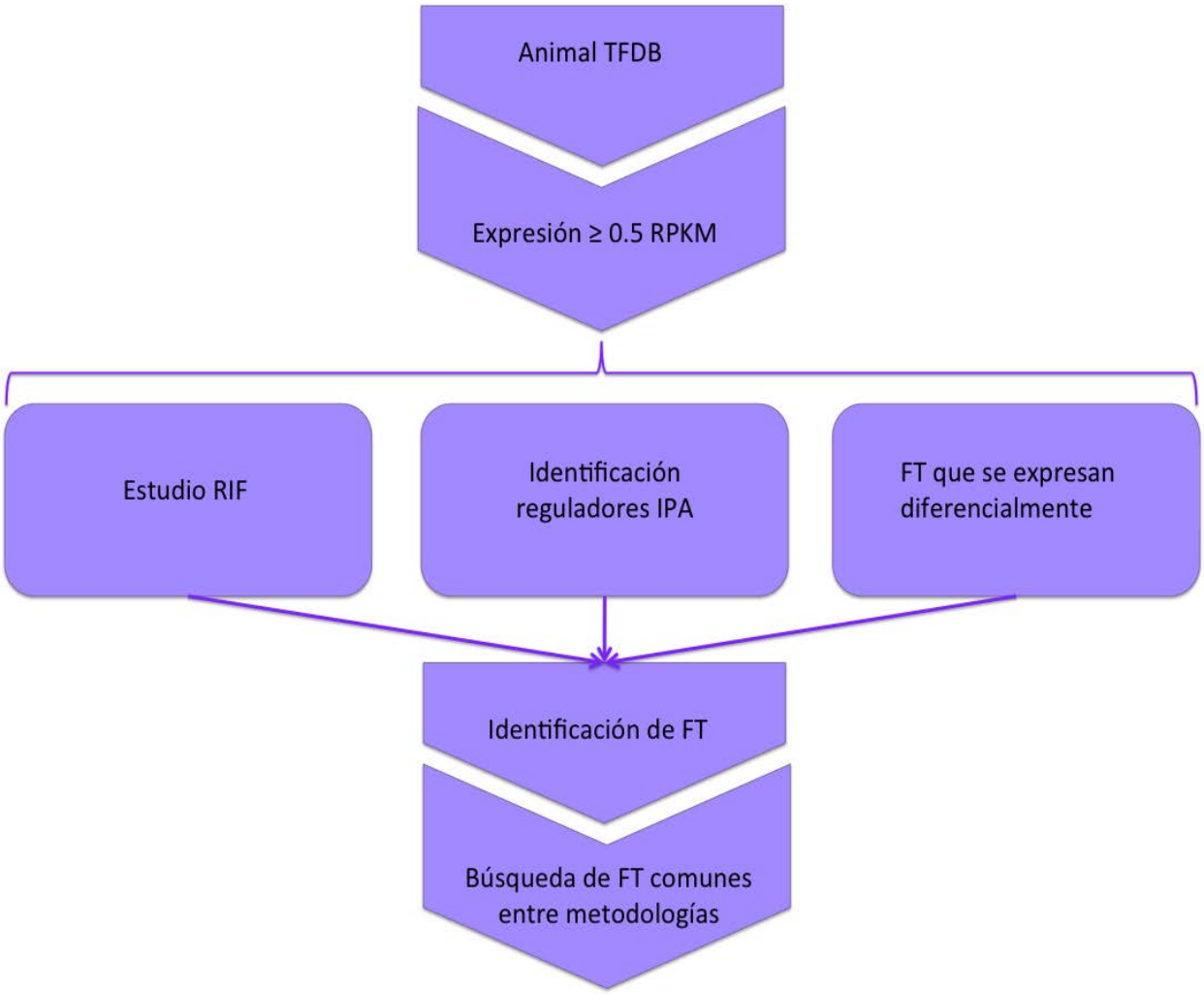
El metabolismo de proteínas parece estar activo en ambos genotipos y músculos, ya que se han identificado numerosos genes relacionados con este proceso. Algunos de estos genes (miembros de la familia de las proteínas de estrés térmico o *heat shock proteins,* HSPs) se expresaban diferencialmente en los dos músculos estudiados. Sin embargo, los cerdos puros mostraron una activación de la proteólisis que no se observó en los cruzados (Capítulo 1, figura 1; capítulo 2 tabla 6). Estos resultados coinciden además con los de estudios previos realizados en ibéricos puros y cruzados (Óvilo et al., 2014) y en cerdo vasco y Large White (Damon et al., 2012), donde el sistema ubiquitina-proteasoma (*ubiquitin proteasome system*, UPS) se encontró activado en las genéticas más grasas, (ibérico puro y cerdo vasco). Rivera-Ferre y colaboradores (2005) observaron, tras comparar cerdos ibéricos y Landrace, una mayor tasa de síntesis proteica y menor desarrollo muscular en el cerdo ibérico, y han sugerido que éste podría tener también una mayor tasa de

degradación de proteína, responsable de su menor capacidad de acumulación de proteína durante el crecimiento (Rivera-Ferre et al., 2005). En este caso, pese a que se trata de dos tipos genéticos muy cercanos, la influencia de genética Duroc podría disminuir la tasa de degradación proteica, lo que se vería reflejado en una menor activación de tal proceso y en un mayor desarrollo muscular, en consonancia con los resultados mostrados en el presente estudio. Además, en animales de 4 meses de edad, algunos de los genes que se encontraron sobreexpreseados en el músculo LD de cerdos ibéricos puros de forma más significativa, están relacionados con la degradación proteica (Capítulo 2, tabla suplementaria 4), de acuerdo a los resultados observados en el nacimiento. Del mismo modo, al nacimiento se encontraron activados procesos relacionados con el sistema inmune en ambos músculos y genotipos, probablemente debido a que los lechones no nacen con un sistema inmune plenamente desarrollado (Becker y Misfeldt, 1993). Sin embargo, a los 4 meses de edad, los cerdos cruzados mostraron un gran número de genes codificantes de inmunoglobulinas altamente sobreexpresados, lo que sugiere una susceptibilidad diferente frente a enfermedades. Este fenómeno se ha descrito entre razas (Sutherland et al., 2005), pero no entre animales de diferente línea paterna.

4.1.3- Genes reguladores potencialmente implicados en los cambios transcripcionales

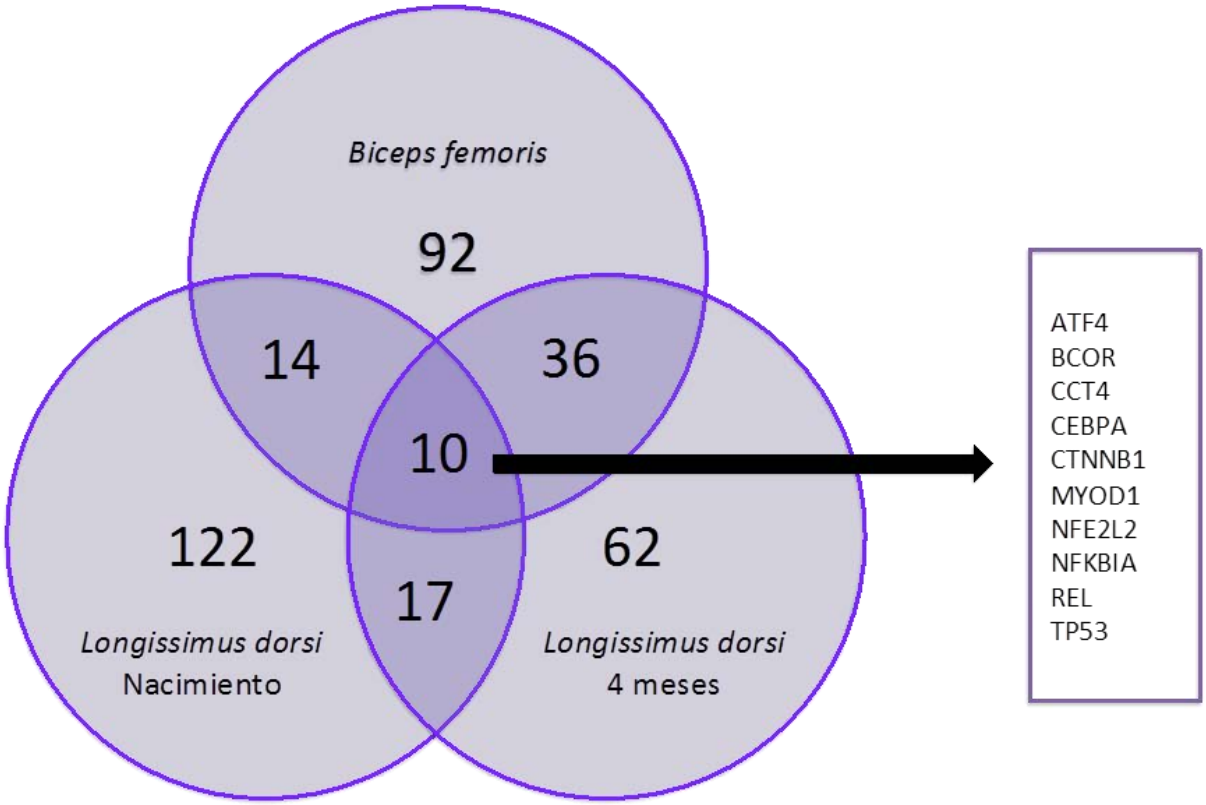
Con el fin de profundizar en el estudio de los mecanismos reguladores que controlan la expresión génica, se realizó un análisis de factores de transcripción mediante distintas metodologías basadas en la bibliografía (software IPA) y en los datos de coexpresión de los genes DE junto con los reguladores recogidos en la base de datos *Animal TFDB* que se expresaban en los tejidos estudiados (*regulatory impact factors, RIF*). También se consideraron factores de transcripción relevantes aquellos que se expresaban diferencialmente entre genotipos (Figura 24).

Figura 24: Esquema que muestra el proceso de identificación y priorización de factores de transcripción (FT).



De esta forma, se combinó información complementaria procedente de distintas fuentes con el fin de identificar factores de transcripción relevantes de la forma más consistente posible, considerándose más robustos aquellos que se evidenciaron siguiendo distintas estrategias. En el músculo LD se identificaron más FT al nacimiento que a los 4 meses de edad (122 frente a 62) (Capitulo 2, tabla suplementaria 5), lo que podría sugerir una regulación más compleja en animales jóvenes. En el músculo BF se identificaron 92 FT (Figura 25). El número de FT evidenciados disminuye de forma notable cuando consideramos sólo los identificados mediante más de un método (15, 3 y 8, en LD al nacimiento, LD a 4 meses y BF, respectivamente). Además, los reguladores identificados en distintas edades o tejidos son de especial interés puesto que su efecto sobre la expresión génica, y por lo tanto sobre las diferencias fenotípicas, parece ser más estable a lo largo de la edad o de la localización anatómica, siendo por lo tanto, potentes genes candidato.

Figura 25: Diagrama de Venn que muestra el número de factores de transcripción identificados en los músculos y edades estudiados, así como los factores de transcripción comunes.



Algunos de estos factores de transcripción son *CEBPA, EGR2, ATFs, PPARGC1B, FOXO1, FOXO3, MEF2D, MYOD1, PVALB* o *SIM1*. Además de haber sido identificados de forma consistente en las distintas muestras analizadas, muchos de estos FT participan en procesos tales como la diferenciación y funcionamiento de células musculares (*MEF2D, MYOD1* y *PVALB* (Edmondson et al., 1992; Murphy et al., 2012; Cho et al., 2015)), el metabolismo proteico (*FOXO3* y *ATFs* (Ebert et al., 2010; Jaitovich et al., 2015)), la diferenciación de adipocitos (*CEBPA, EGR2, FOXO1* y *ATFs*, (Boyle et al., 2009; Maekawa et al., 2010; Gupta et al., 2013)) o el metabolismo lipídico y enfermedades relacionadas tales como la obesidad o la diabetes (*PPARGC1B, SIM1* (Franks et al., 2014; Tolson et al., 2014; Villegas et al., 2014)). Estos procesos determinan en gran medida las diferencias observadas a nivel fenotípico en parámetros productivos y de calidad de carne entre cerdos ibéricos puros y cruzados. Además, su papel en dichas funciones puede ser extrapolable a distintas especies, por lo que su identificación en cerdos de genética ibérica, que han demostrado ser un buen modelo para el estudio de enfermedades relacionadas con el metabolismo lipídico (Torres-Rovira et al., 2012), puede ser de interés también en investigación clínica humana.

Finalmente, aprovechando la información de secuencia que proporciona la técnica RNA-Seq se ha llevado a cabo una búsqueda de variantes estructurales entre todos los genes expresados en el

músculo BF al nacimiento. Además de esto, se realizó un segundo análisis de polimorfismos más detallado en algunos de los reguladores considerados de interés, debido a su potencial impacto sobre el fenotipo. Entre ellos, *PPARGC1B* y *TRIM63* fueron los más polimórficos, Muchas de las variantes identificadas podrían causar alteraciones en la función de las proteínas. Entre las variantes estructurales identificadas en *PPARGC1B* cabe destacar dos con potencial repercusión en la proteína codificada: una mutación puntual no sinónima y una delección de un codon completo. Estas variantes resultan de gran interés puesto que previamente se han descrito polimorfismos en este gen asociados con diabetes tipo 2 y con cambios en la acumulación de grasa subcutánea en humanos (Franks et al., 2014; Villegas et al., 2014). Por otro lado, se identificaron 15 mutaciones no sinónimas y otras 7 que modifican el marco de lectura en el gen *TRIM63*, que regula la homeostasis de las proteínas en el músculo, favoreciendo su degradación mediante el sistema UPS (Chen et al., 2012). El estudio de los mecanismos moleculares y genéticos que determinan la cantidad y composición de la GIM aporta un conocimiento básico que podría ser aplicable a la industria a medio-largo plazo. Sin embargo, debido a la gran importancia de la GIM sobre la calidad de la carne también es importante buscar estrategias que permitan, a corto plazo, modificar estos parámetros.

4.2- EFECTO DEL NIVEL DE INCLUSIÓN DE VITAMINA A EN LA DIETA SOBRE ASPECTOS PRODUCTIVOS Y DE CALIDAD DE CARNE Y SOBRE LA EXPRESIÓN GÉNICA EN CERDOS IBÉRICOS PUROS.

Las estrategias nutricionales basadas en la modificación del contenido de macronutrientes han demostrado tener un impacto variable sobre el engrasamiento en porcino (Weber et al., 2006), resultando en general menos afectado el compartimento intramuscular que el subcutáneo (Suarez-Belloch et al., 2013) lo que frecuentemente se traduce en canales con exceso de grasa dorsal y por tanto, poco interesantes para la industria. Esto probablemente se debe a que estas estrategias (modificación de la energía, adición de distintos tipos de grasa o alteración de la relación energía:proteína) estimulan la lipogénesis (Wolfe et al., 1977; Bee et al., 2002; Gondret y Lebret, 2002; Tan et al., 2011; Castellano et al., 2015), es decir, la acumulación lipídica en los adipocitos maduros presentes. Debido a la mayor concentración de adipocitos en la grasa subcutánea, parece lógico que éste sea el principal depósito afectado. Por ello un mecanismo de acción más apropiado podría ser la estimulación de la adipogénesis en los preadipocitos y células indiferenciadas intramusculares. Puesto que su desarrollo es más tardío que el de las células grasas subcutáneas (que se diferencian en la etapa fetal) y se mantiene activo en animales jóvenes (Gondret et al., 2008; Poulos et al., 2010), los preadipocitos intramusculares pueden ser más susceptibles a estímulos proadipogénicos durante fases tempranas del crecimiento postnatal. De este modo, ciertas estrategias aplicadas en distintas fases del crecimiento postnatal podrían conseguir aumentar la

cantidad de GIM sin modificar la grasa subcutánea. Se ha observado en cultivos celulares que la VA, en concreto su principal metabolito activo el AR, añadida al medio de cultivo inhibe la diferenciación e incluso favorece la apoptosis de preadipocitos (Sato y Hiragun, 1988; Suryawan y Hu, 1997; Kim et al., 2000). Sin embargo, sus efectos *in vivo* no son consistentes. En el cerdo, se han obtenido resultados contradictorios que además podrían estar condicionados por el genotipo de los animales (D'Souza et al., 2003; Olivares et al., 2009a; Olivares et al., 2009b; Olivares et al., 2011). Es por ello que son necesarios más estudios que esclarezcan los efectos de la VA en distintas razas de interés, como el cerdo ibérico.

En el presente trabajo se ha estudiado el efecto de la restricción de VA, iniciada en dos momentos distintos del desarrollo (2 (ER) y 4 (LR) meses de edad) sobre aspectos productivos y de calidad de carne en cerdos de 4, 8 y 11 meses de edad. Los parámetros estudiados fueron la cantidad y composición de GIM y subcutánea, así como la concentración tisular de vitamina A y E y la expresión de genes candidato involucrados en el metabolismo de la VA, la diferenciación de adipocitos y la lipogénesis.

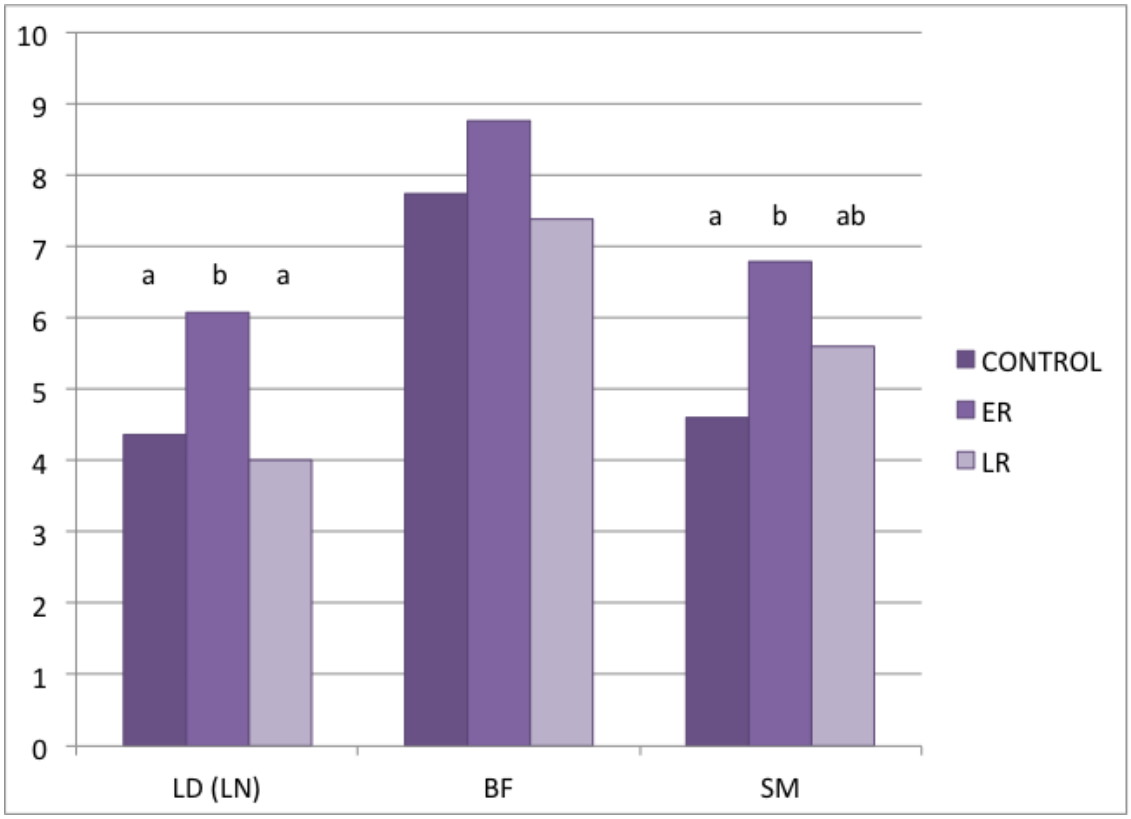
4.2.1- Efecto del nivel de inclusión de vitamina A en la dieta sobre aspectos productivos y de calidad de carne en cerdos ibéricos puros.

La restricción de VA en la dieta produjo un retraso en el crecimiento y un empeoramiento en el índice de conversión durante el período de crecimiento que podría ser debido a que la restricción se aplicó desde una edad muy temprana (Capítulo 3, tabla 3). Sin embargo este efecto no se vio reflejado en el rendimiento ni peso finales, de acuerdo a la bibliografía previa (D'Souza et al., 2003; Olivares et al., 2009a; Olivares et al., 2009b; Olivares et al., 2011).

Cuando la VA se administra en la dieta en cantidad suficiente (como ocurre en el grupo control, suplementado con 10,000 UI de VA/kg de pienso), ésta se acumula en dos formas químicas principalmente, la forma alcohol (retinol) y la forma éster (palmitato de retinol), que es la mayoritaria (D'Ambrosio et al., 2011). En el presente estudio la cantidad de palmitato de retinol acumulada en los tejidos fue 12 veces superior a la de retinol (Capítulo 3, figura 1). Además, la VA se acumula preferentemente en el hígado (Blaner, 1994), mostrando éste niveles de retinol hasta 70 veces superiores que los encontrados en la grasa tras 9 meses de suplementación. La evolución de los depósitos de VA se evaluó a lo largo de la vida del animal, observándose que durante la fase de crecimiento (desde 35.8 hasta 101.4 kg de PV), la acumulación fue mayor que durante la fase final del cebo, lo que podría estar relacionado con una saturación en los depósitos tras un período largo de suplementación. En los grupos restringidos (ER y LR), por otro lado, se apreció una movilización de los depósitos de VA desde el inicio de la restricción, que se produjo de forma más marcada que la acumulación en todos los depósitos y que fue más evidente en el caso del retinol, en

concordancia con los resultados de un estudio previo en cerdos (Olivares et al., 2009c). Además, este proceso parece ser selectivo, siendo el hígado el primer depósito en movilizar el retinol acumulado, del mismo modo que se ha descrito en vacuno (Pickworth et al., 2012). Esta movilización parece estar influida por la edad a la que se inicia la restricción, puesto que los animales LR sufrieron una depleción de los depósitos tres veces superior a la observada en los animales ER (Capítulo 3, tabla 3). Estos resultados podrían sugerir la existencia de un mecanismo de control de la movilización en función de la acumulación tisular de VA previa, mayor en el grupo LR. Asociado a la retirada de VA de la dieta, además de la disminución de su concentración tisular, se observó un aumento del contenido de α-tocoferol o vitamina E (reconocido por su efecto positivo sobre la calidad de la carne (Dirinck et al., 1996)) tanto en hígado como en grasa. Cabe destacar que este aumento se produjo de forma más marcada en el caso del grupo LR en los depósitos grasos, por lo que se podría establecer que, con el fin de aumentar el contenido de α-tocoferol en la grasa subcutánea, es más eficaz iniciar la restricción a una edad ligeramente más tardía. De hecho, en un estudio previo se observó que la retirada de VA de la dieta durante las 5 últimas semanas de cebo aumentaba la concentración tisular de α-tocoferol (Olivares et al., 2009c). Además de sus efectos sobre la acumulación de α-tocoferol, la restricción de VA afectó a parámetros de calidad de carne. Debido a su efecto antiadipogénico, la retirada de VA en la dieta, en fases iniciales de crecimiento (35.8 kg PV), produjo un aumento del 73% en el número de preadipocitos en el músculo LD de cerdos ER (Capítulo 4, figura 1). Este incremento en el número de células precursoras podría implicar un mayor potencial de acumulación de grasa y por lo tanto, podría relacionarse con un mayor contenido en GIM en adultos (Hausman et al., 2014). En concordancia con esta idea, en el presente estudio se observó que en animales al sacrificio (158 kg PV) el contenido en LN (la fracción mayoritaria de la GIM) del músculo LD, así como la GIM total en el músculo SM fueron superiores (34% y 47%, respectivamente) en los animales sometidos a una restricción de VA en la dieta desde los dos meses de edad (Figura 26). Sin embargo, el músculo BF no presentó diferencias entre los distintos grupos (Capítulo 5, tabla 5). El contenido en GIM de este músculo fue mayor que en el resto de los estudiados y se encuentra entre los niveles más altos descritos en la bibliografía (Muriel et al., 2004; Ventanas et al., 2006; Fuentes et al., 2014). Estas diferencias en la capacidad adipogénica y de acumulación de lípidos entre distintos músculos, están en concordancia con los resultados hallados en los estudios de RNA-Seq incluidos en el capítulo 2. Es posible que en un músculo con niveles de grasa tan elevados, sea más difícil aumentar este parámetro mediante estrategias nutricionales.

Figura 26: Grasa intramuscular en los músculos *Longissimus dorsi* (LD), *Biceps femoris* (BF) y *Semimembranosus* (SM) a 158 kg de peso vivo.



LD: músculo *Longissimus dorsi*; LN: Lípidos neutros; BF: músculo *Biceps femoris*; SM: músculo *Semimembranosus*; CONTROL: Cerdos que recibieron 10,000 UI vitamina A/kg pienso durante todo el periodo; ER: Cerdos que recibieron un pienso no suplementado con vitamina A desde los dos meses de edad; LR: Cerdos que recibieron un pienso no suplementado con vitamina A desde los cuatro meses de edad; Las letras *a* y *b* indican diferencia estadísticamente significativa.

En cuanto a la composición de la GIM, los efectos observados en cerdos jóvenes han sido de escasa magnitud. Sin embargo, cuando los cerdos alcanzaron la edad de sacrificio (158 kg PV), se observó un aumento de los AGMI en detrimento de los AGS en los animales restringidos, y por lo tanto un incremento del índice de desaturación AGMI/AGS de forma consistente, tanto en la GIM de los tres músculos estudiados, como en la grasa subcutánea dorsal y del jamón (Capítulo 4, tabla 5; capítulo 5, tablas 4 y 5). También se observó un descenso en el ratio n6/n3 en los dos grupos de animales restringidos respecto al control, que tiene efectos potencialmente positivos sobre la salud del consumidor (Who y Consultation, 2003). El hígado fue el único tejido no afectado por el tratamiento (Capítulo 5, tabla 6), de forma similar a los resultados observados previamente en cerdos de genéticas grasa y magra (Olivares et al., 2009a; Olivares et al., 2011). Hasta ahora no se había descrito un efecto tan marcado y consistente del aporte de VA sobre el perfil de ácidos grasos en distintos tejidos del cerdo. En concreto, la GIM parece ser menos sensible que la grasa subcutánea especialmente en razas de cerdo magras (Olivares et al., 2009a; Olivares et al., 2011), lo que explicaría por qué en el presente estudio, desarrollado en una línea de cerdos muy grasa

encontramos estos resultados tan claros en los distintos depósitos (tanto subcutáneo como intramuscular). No sólo los niveles de VA administrada en el pienso, sino también la edad modificaron el perfil de ácidos grasos. La concentración de AGS disminuyó con la edad, mientras que la de AGMI y AGPI aumentaron a lo largo de las tres edades estudiadas (Capítulo 5, figura 1), de forma similar a los resultados obtenidos por Daza y colaboradores (2007). Este hecho, es además coherente con lo observado en el experimento 1, en el que los animales recién nacidos de ambas genéticas presentaron niveles inferiores de AGMI que los animales de 4 meses de edad en la GIM (Capítulo 2, tabla 2). En el mismo período, se observó una ligera disminución con la edad de los AGS más evidente en los animales cruzados y de AGPI en ambos tipos genéticos. La evolución del perfil de ácidos grasos con la edad es especialmente relevante en el caso de los productos cárnicos del cerdo ibérico, donde se busca una alta concentración de AGMI (Ventanas et al., 2006).

4.2.2- Efecto del nivel de inclusión de vitamina A en la dieta sobre la expresión génica en cerdos ibéricos puros.

El AR es un potente regulador de la expresión génica (Balmer y Blomhoff, 2002), y los efectos de la restricción de VA sobre el crecimiento y la composición de los tejidos están probablemente mediados por cambios transcripcionales debidos principalmente a la unión del retinol con distintos factores de transcripción. Por ello, se evaluó la expresión de una serie de genes candidato en distintas edades y tejidos según el rol en el que cada gen está implicado. En primer lugar, en animales jóvenes (35.8 kg PV) se valoró la expresión de genes relacionados con la diferenciación de adipocitos en el músculo LD. A pesar del efecto señalado sobre el número de preadipocitos que supuso la restricción de VA, sólo se detectó una tendencia en el gen *CRABPII*, cuya inhibición se ha asociado con el proceso de diferenciación (Berry et al., 2010), lo que concuerda con la menor expresión génica observada en los animales restringidos (Capítulo 4, figura 2). Por otro lado, para identificar genes relacionados con los cambios de composición observados en los animales adultos, se realizó un estudio de expresión génica de genes relacionados con el control de la adipogénesis, la síntesis y metabolismo de ácidos grasos, la homeostasis energética y la señalización de la VA en animales control y ER de 101.4 kg PV, puesto que se consideró que dichos cambios debían preceder temporalmente a los efectos fenotípicos observados. De los 18 genes analizados en el hígado, *ACOX1, CEBPB* e *IGF1* mostraron mayor expresión en los animales ER (Capítulo 5, figura 2A). El gen *ACSL4*, involucrado junto con *ACOX1* en el metabolismo de ácidos grasos, mostró una tendencia en el mismo sentido. Por otro lado, los genes *IGF1* y *CEBPB* están relacionados con la diferenciación de adipocitos (Wabitsch et al., 1995; Yeh et al., 1995). Schwarz y colaboradores (1997) describieron cómo los efectos antiadipogénicos del AR están mediados por la interrupción de la activación de genes diana por parte del gen *CEBPB*. Esto, unido a la mayor expresión observada en el grupo ER

confirma el papel que este gen juega en los efectos del AR sobre la adipogénesis, que no sólo dependerían de una alteración en su función, sino también en la expresión del gen *CEBPB*. También se analizó la expresión 19 genes en el tejido adiposo, encontrándose *CRABPII* y *SCD* sobreexpresados en el grupo ER y *RXRG* en el grupo control (Capítulo 5, figura 2B). El gen *SCD* codifica la enzima Delta-9-desaturasa, que cataliza el paso de AGS a AGMI (Paton y Ntambi, 2009). Existen resultados contradictorios sobre la regulación del gen *SCD* por la VA (Miller et al., 1997; Daniel et al., 2004; Siebert et al., 2006; Arnett et al., 2007; Gorocica-Buenfil et al., 2007a; Olivares et al., 2009b). En el presente trabajo, la VA reguló negativamente su expresión, siendo ésta mayor en el grupo ER, lo cual es coherente con el aumento en la concentración de AGMI en detrimento de los AGS en este grupo. El gen *CRABPII* también se encontró sobreexpresado en el grupo ER. Este gen codifica una proteína transportadora de AR desde el citoplasma al núcleo (Takase et al., 1986; Dong et al., 1999), por lo que su activación en los animales restringidos podría deberse a un mecanismo de regulación homeostática para facilitar la señalización del AR, probablemente escaso en estos cerdos. Por otro lado, es importante señalar que se trata de una proteína que sólo se expresa en preadipocitos, no en adipocitos maduros, puesto que como se ha comentado previamente, su inhibición es un paso necesario en el proceso de diferenciación. Por ello, es posible que la mayor expresión de este gen en el grupo ER esté asociado con una mayor presencia de preadipocitos en estos animales que aún están en fase de crecimiento tardío y que, por lo tanto, pueden disponer de células adiposas indiferenciadas (Margareto et al., 2001). Finalmente, el gen *RXRG*, sobreexpresado en el grupo control, codifica para un receptor nuclear con afinidad para uno de los isómeros del AR, *9-cis-AR* (Bonet et al., 2003). Los mecanismos de regulación de la expresión de este tipo de receptores son muy complejos y no se conocen por completo. En este trabajo se presentan nuevas evidencias que indican una regulación positiva de la VA sobre la expresión de uno de sus receptores nucleares, *RXRG*. Los niveles de VA en la dieta afectaron también a la expresión de genes que regulan su propio metabolismo, aumentando la expresión de genes como *ADH1C*, *ALDH1A1* o *LRAT*, que muestran un patrón de expresión dosis-dependiente, de acuerdo con estudios previos realizados en ratas (Ross y Zolfaghari, 2004). La mayor expresión de estos genes en animales suplementados podría ser consecuencia de un mecanismo de regulación que permite mantener niveles constantes de retinol y AR en el organismo mediante su oxidación y esterificación, ya que niveles altos de retinol podrían superar el umbral de toxicidad, especialmente en animales adultos (Molotkov y Duester, 2003). El gen RBP4, que codifica una proteína liberada al torrente sanguíneo únicamente en su forma unida al retinol, presentó una expresión similar entre ambos grupos, de acuerdo con el mantenimiento de la homeostasis del retinol previamente comentado. El nivel de VA en la dieta no produjo ningún efecto sobre la expresión de genes involucrados en el metabolismo y transporte del α-tocoferol. En lo que respecta al efecto del tiempo sobre la expresión de los genes relacionados con el metabolismo de la VA y α-tocoferol, los genes relacionados con la VA mostraron

mayor expresión a los 101 kg de PV, mientras que los genes involucrados en el metabolismo de la vitamina E mostraron menor expresión en este periodo que durante el crecimiento temprano (35.8 kg) y la fase final del cebo (158 kg) (Capítulo 3, figura 4). Este efecto de la edad sobre la expresión génica podría determinar la respuesta a la restricción de VA y debería por tanto ser considerado al diseñar programas de alimentación que incluyan etapas de restricción de VA o de suplementación con vitamina E, prácticas cada vez más frecuentes en alimentación porcina.

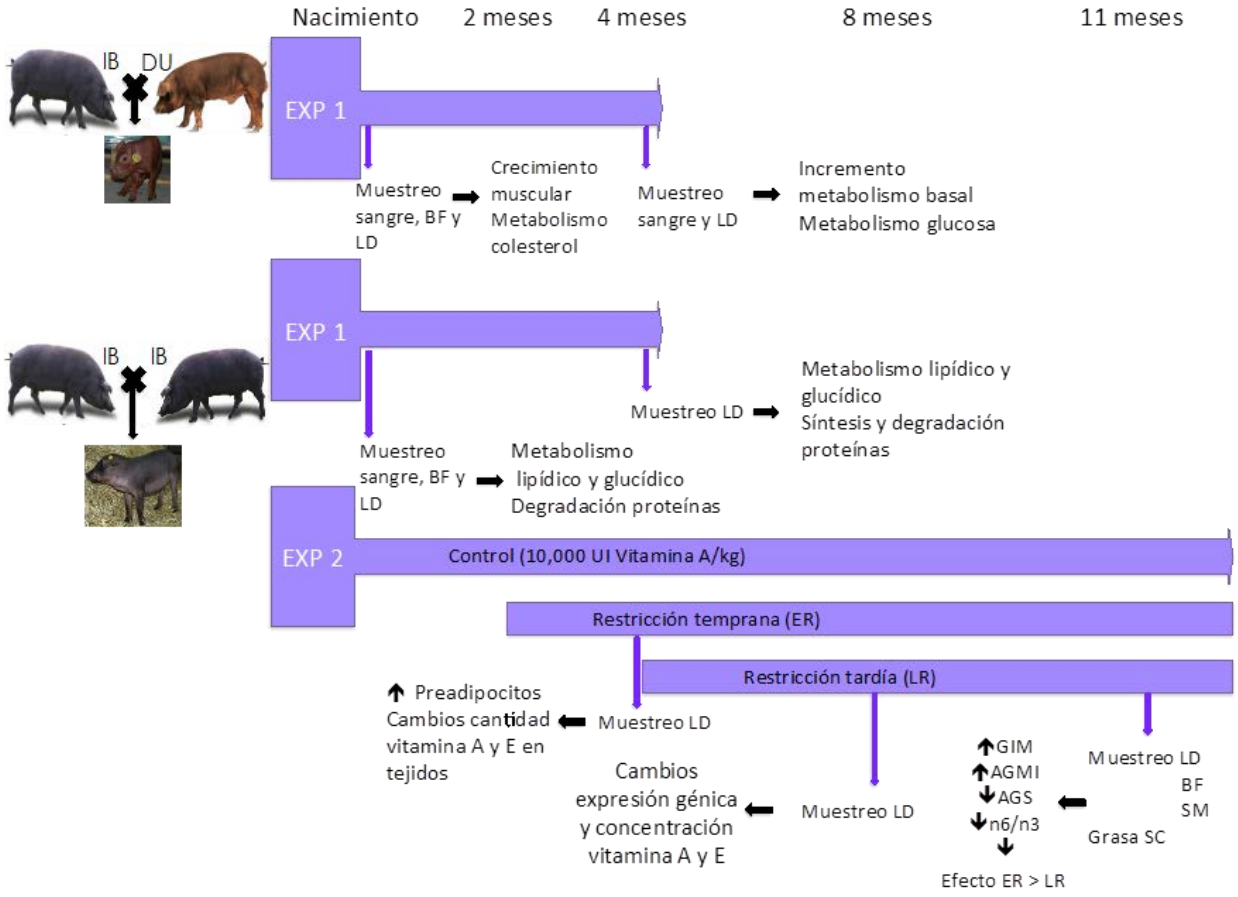
Pese a que los efectos sobre la adipogénesis se observaron a edades tempranas (35.8 kg PV) (Capítulo 4, figura 1), resulta llamativa la ausencia de efecto de la VA sobre el contenido y composición de la GIM en animales en crecimiento, mientras que este efecto es claramente detectable al final del cebo (Capítulo 5, tablas 4 y 5). Esto pone de manifiesto la importancia de ambos procesos, hiperplasia e hipertrofia (que se produce en estadios posteriores), en el desarrollo de los depósitos grasos, así como el papel que desempeña la VA en la regulación de ambos. Además, teniendo en cuenta que estos efectos parecen ser de pequeña magnitud y acumulativos, es necesario un tiempo suficiente de restricción para que tenga lugar la depleción de las reservas de VA, necesaria para generar un nivel de VA suficientemente bajo que permita provocar cambios en la expresión génica que a su vez alteren las rutas metabólicas y enzimáticas, dando lugar a los cambios observados en la composición de la carne. Por otro lado, es preciso tener en cuenta que las materias primas de los piensos contienen niveles de VA que en algunos casos pueden ser relevantes, por lo que aunque la ración no esté suplementada, el animal recibe un aporte basal que podría condicionar los niveles tisulares y por tanto la efectividad de la restricción.

4.3- CONSIDERACIONES FINALES

En conjunto, la presente Tesis Doctoral proporciona información básica y aplicada sobre mecanismos genéticos y nutrigenómicos que influyen en el desarrollo de los depósitos grasos, el crecimiento y el metabolismo energético en cerdos ibéricos puros y cruzados durante su crecimiento (Figura 27). Como se ha observado en el estudio del tipo genético, el nacimiento es un momento de gran activación de procesos relacionados con el crecimiento y la diferenciación celular, mientras que a los 4 meses de edad, los procesos proliferativos son menos importantes y el mantenimiento de la homeostasis se muestran como funciones predominantes. Si consideramos que el mecanismo fundamental por el que la restricción de VA modifica el contenido en GIM consiste en alterar la diferenciación adipocitaria, los resultados del estudio transcripcional sugieren una mayor susceptibilidad en periodos próximos al nacimiento. En concordancia, los resultados observados en esta Tesis demuestran que la efectividad es mayor cuando la restricción de VA se inicia en las primeras edades. En conjunto, estos resultados sugieren que el periodo postnatal entre

el nacimiento y los dos meses de edad es crítico para el desarrollo de los adipocitos intramusculares, mientras que a los cuatro meses, los procesos de diferenciación celular parecen ser menos activos, lo que explicaría la menor respuesta a la restricción de VA observada en estos animales (Figura 26).

Figura 27: Diseño experimental, muestras analizadas y principales resultados obtenidos en cada edad.



La estrategia de restricción de VA supone un pequeño ahorro en el coste del pienso (alrededor de 0.04%) y supone ventajas como el aumento de la concentración α-tocoferol, GIM y AGMI en detrimento de los AGS en los tejidos y podría ser de interés en muchos otros ámbitos de la producción porcina, ya que favorece el desarrollo de carnes con mejores atributos de calidad. Por otra parte los cerdos ibéricos cruzados con Duroc, presentan diferencias tanto en contenido y composición de la GIM como a nivel metabólico y transcripcional ya desde edades muy tempranas, como se ha observado en el experimento 1. Por ello la eficacia de la estrategia de restricción de VA debería estudiarse específicamente en este tipo genético, ya que el periodo crítico de restricción y las consecuencias metabólicas de la misma podrían variar entre genotipos. Además, en caso de

validarse los resultados observados en animales puros, la restricción de VA podría ser una herramienta eficaz para homogeneizar y mejorar la calidad de la carne en los animales cruzados, consiguiendo características más similares (mayor contenido en GIM, en AGMI y menor ratio n6/n3) a las encontradas en cerdos ibéricos puros.

Este trabajo demuestra que la nutrigenómica es una disciplina esencial para la comprensión de los efectos fisiológicos y metabólicos en el diseño de programas de alimentación. Esta disciplina aporta información básica con potencial utilidad práctica, ya que puede permitir optimizar la respuesta biológica a distintos tipos de tratamientos nutricionales.

Las prácticas habituales de suplementación con vitaminas liposolubles durante toda la etapa de crecimiento deberían ser reconsideradas a la vista de nuestros resultados. Esto es debido a la existencia de antagonismos y periodos críticos de diferenciación celular, que determinan las necesidades de aporte o restricción vitamínicas durante períodos concretos. El tipo genético, el músculo diana y el objetivo productivo deberán ser también considerados. Esta situación abre las puertas a un importante campo de investigación que permita conocer la respuesta a cada nutriente en cada momento del ciclo productivo y desarrollar nuevas recomendaciones nutricionales más eficientes y "a la carta" según los objetivos productivos. En este sentido, es necesario tener en cuenta que las nuevas herramientas de análisis (ómicas) permiten abordar el campo de la genética y del metabolismo de una forma global. Por otro lado, la disponibilidad de herramientas de genotipado masivo cada vez más asequibles abren la puerta al estudio de la respuesta a los nutrientes condicionado por el genotipo (nutrigenética), que podrían conducir idealmente a recomendaciones nutricionales "individualizadas" o condicionadas por el genotipo.

El desarrollo de la ciencia hace confluir a los distintos campos científicos y obliga al diseño de proyectos de investigación no sólo multidisciplinarios, sino interdisciplinarios, puesto que es la interacción de las disciplinas la que favorece el conocimiento.

5.-CONCLUSIONES/CONCLUSIONS

CONCLUSIONES

1. La tecnología RNA-Seq aplicada al tejido muscular posibilita la obtención de resultados robustos para la comprensión de los mecanismos reguladores implicados en el desarrollo y la calidad de la carne en el cerdo.

2. La edad afecta a la expresión de un gran número de genes y rutas metabólicas, caracterizándose la etapa neonatal por fenómenos de biosíntesis y diferenciación celular y el periodo juvenil por procesos catabólicos y homeostáticos. Además la grasa intramuscular aumenta con la edad y la composición de ácidos grasos se modifica, tanto en la grasa intramuscular como en la subcutánea, aumentando la concentración de ácidos grasos monoinsaturados en detrimento de los saturados.

3. El músculo *Biceps femoris* y *Longissimus dorsi* muestran diferencias en el metabolismo y potencial de acumulación de grasa que pueden estar relacionadas con su diferente capacidad de respuesta al efecto de la genética y de la nutrición.

4. La inclusión de genética Duroc (50%) resulta en lechones de mayor peso y tamaño al nacimiento que, sin embargo, son igualados por los cerdos puros en etapas posteriores del desarrollo. Los niveles plasmáticos de colesterol y triglicéridos muestran un efecto similar.

5. Los estudios del transcriptoma ponen de manifiesto que el metabolismo lipídico y de la glucosa es más activo en los cerdos ibéricos puros a lo largo del desarrollo, mientras que los cruzados presentan una predisposición mayor para el crecimiento muscular y el gasto energético. Sin embargo, a los cuatro meses de edad, los cerdos puros presentan activación de algunos procesos relacionados con el crecimiento, como el metabolismo de aminoácidos o la proliferación celular, que podrían relacionarse con el crecimiento compensatorio postnatal experimentado por estos animales.

6. Los resultados de expresión génica indican también que la degradación proteica es un proceso importante en ambos genotipos, pero de forma más evidente en ibéricos puros, de acuerdo con la mayor tasa de degradación proteica descrita en esta raza, que explicaría sus diferencias en potencial de crecimiento.

7. La estrategia propuesta para la identificación de genes reguladores, combinando metodologías complementarias (basadas en la bibliografía y en los datos de expresión diferencial y de

coexpresión) permite identificar y priorizar factores de transcripción de forma objetiva y robusta. Algunos de los reguladores más importantes identificados son *PPARGC1B*, *TRIM63*, *EGR2*, *FOXO1*o *PVALB*.

8. Existe una gran cantidad de polimorfismos en los genes *PPARGC1B* y *TRIM63*, muchos de los cuales provocan cambios aminoacídicos que podrían alterar sus funciones en la regulación del gasto energético, la oxidación de la grasa o el metabolismo proteico.

9. La administración de niveles suficientes de vitamina A en la dieta favorece su acumulación en los tejidos, principalmente en el hígado en forma de palmitato de retinol. Los cerdos sometidos a una restricción en el aporte de vitamina A responden con una rápida movilización de dichos depósitos, lo que favorece la acumulación de α-tocoferol en los tejidos. La efectividad es mayor cuando la restricción comienza a los cuatro meses de edad.

10. La retirada de la vitamina A de la dieta a los dos meses de edad aumenta el número de preadipocitos en un 73% en cerdos de cuatro meses de edad, así como el contenido en lípidos neutros en el músculo *Longissimus dorsi* y de grasa intramuscular en el *Semimembranosus* en cerdos adultos. Estos cambios no están acompañados por un aumento de la grasa subcutánea ni por efectos negativos sobre el rendimiento productivo.

11. La restricción de vitamina A produce en todos los tejidos analizados excepto el hígado, un aumento de la concentración de ácidos grasos monoinsaturados en detrimento de los saturados, y un aumento en la expresión del gen *SCD*. Asimismo, produce un descenso del ratio n6/n3 en los cerdos restringidos. El efecto de la restricción es más marcado cuando ésta comienza a los dos meses de edad.

12. Los efectos de la vitamina A sobre la composición de la carne están acompañados por cambios en la expresión de genes involucrados en la diferenciación de adipocitos (*IGF1* y *CEBP*), en el metabolismo de ácidos grasos (*ACOX*, *ACSL4*, *SCD*) y en el metabolismo y señalización del retinol(*RXRG*, *CRABPII*, *ADH1C*, *LRAT*).

CONCLUSIONS

1. RNA-Seq technology applied to muscle tissue provides robust results for the understanding of regulatory mechanisms involved in development and meat quality in the pig.

2. Age affects the expression of a large number of genes and pathways, the neonatal period being characterized by biosynthetic and cellular differentiation events and the juvenile period by homeostatic and catabolic processes. Moreover, intramuscular fat content increases with age and fatty acids composition is modified both in intramuscular and subcutaneous depots, with monounsaturated increasing at the expense of saturated fatty acids.

3. Biceps femoris and Longissimus dorsi muscles show differences in metabolism and fat accumulation potential that may be related to their different response to genetic and nutritional effects.

4. The inclusion of Duroc genetics (50%) results in heavier and bigger piglets at birth, which are reached by purebred pigs in later growing stages. Plasma cholesterol and triglycerides levels show a similar effect.

5. Transcriptomic studies reveal that lipid and glucose metabolism is more active in purebred Iberian pigs along development, while crossbred pigs show better predisposition for muscular growth and increased energy expenditure. However, at four months of age, purebred Iberian pigs manifest an activation of some processes associated with growth, such as aminoacids metabolism or cellular proliferation, which may be related to the postnatal catch-up growth experienced by these animals.

6. Gene expression results indicate that protein degradation is an important process in both genotypes, but it is even more evident in purebred Iberians, in agreement with the higher protein degradation rate reported in this breed, which would explain their differences in growth potential.

7. The proposed strategy for identifying regulator genes, combining complementary methodologies (based in previous literature and in differential expression and coexpression data) allows identifying and prioritizing transcription factors in a robust and objective manner.

Some of the most important regulators identified are *PPARGC1B, TRIM63, EGR2, FOXO1* or *PVALB*.

8. A large amount of polymorphism exists in genes *PPARGC1B* and *TRIM63*, several of them causing aminoacid changes which may alter their functions in regulating the energy expenditure, fat oxidation or protein metabolism.

9. The administration of sufficient dietary vitamin A levels promotes its tissue accumulation, mainly in liver as retinyl palmitate. Pigs subjected to dietary vitamin A restriction show a fast depots mobilization, which facilitates tissue α-tocopherol accumulation. Efficiency is higher when restriction starts at four months of age.

10. The withdrawal of dietary vitamin A from two months of age increases the preadipocyte number by 73% in four months old pigs, as well as the neutral lipid in *Longissimus dorsi* and the intramuscular fat content in *Semimembranosus* muscle in adult pigs. These changes are not accompanied by increases in subcutaneous fat or by negative effects on productive performance.

11. Dietary vitamin A restriction leads to an increase in monounsaturated at the expense of saturated fatty acids and increase in *SCD* gene expression in all analyzed tissues except in liver. Also, it decreases the n6/n3 ratio in restricted pigs. The effect of vitamin A restriction is more marked when it starts from two months of age.

12. The effects of dietary vitamin A on meat composition are accompanied by expression changes of genes involved in adipocyte differentiation (*IGF1* and *CEBP*), fatty acids metabolism (*ACOX*, *ACSL4* and *SCD*) and retinol signaling and metabolism (*RXRG*, *CRABPII*, *ADH1C* and *LRAT*).

6.- REFERENCIAS

Aagaard-Tillery, K.M., Grove, K., Bishop, J., Ke, X., Fu, Q., McKnight, R., Lane, R.H., 2008. Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. Journal of molecular endocrinology 41, 91-102.

Abawi, F., Sullivan, T., 1989. Interactions of vitamins A, D3, E, and K in the diet of broiler chicks. Poultry Sci 68, 1490-1498.

Adzhubei, I.A., Schmidt, S., Peshkin, L., Ramensky, V.E., Gerasimova, A., Bork, P., Kondrashov, A.S., Sunyaev, S.R., 2010. A method and server for predicting damaging missense mutations. Nat. Methods 7, 248-249.

Ahmed, F., Jones, D.B., Jackson, A.A., 1990. The interaction of vitamin A deficiency and rotavirus infection in the mouse. British journal of nutrition 63, 363-373.

Ailhaud, G., Hauner, H., 1998. Development of white adipose tissue, In: Bray, G., Bouchard, C., James, W. (Eds.), Handbook of obesity, Marcel Dekker Inc,, New York, pp. 359-378.

Alam, S.Q., Alam, B.S., Ta-Wei, C., 1984. Activities of fatty acid desaturases and fatty acid composition of liver microsomes in rats fed β-carotene and 13-cis-retinoic acid. Biochim. Biophys. Acta 792, 110-117.

Alapatt, P., Guo, F., Komanetsky, S.M., Wang, S., Cai, J., Sargsyan, A., Díaz, E.R., Bacon, B.T., Aryal, P., Graham, T.E., 2013. Liver retinol transporter and receptor for serum retinol-binding protein (RBP4). J. Biol. Chem. 288, 1250-1265.

Alexander, D.L., Ganem, L.G., Fernandez-Salguero, P., Gonzalez, F., Jefcoate, C.R., 1998. Aryl-hydrocarbon receptor is an inhibitory regulator of lipid synthesis and of commitment to adipogenesis. J. Cell Sci. 111, 3311-3322.

Alexandre, K., Smit, A., Gray, I., Crowther, N., 2008. Metformin inhibits intracellular lipid accumulation in the murine pre- adipocyte cell line, 3T3- L1. Diabetes, Obesity and Metabolism 10, 688-690.

Allen, D.L., Unterman, T.G., 2007. Regulation of myostatin expression and myoblast differentiation by FoxO and SMAD transcription factors. American Journal of Physiology-Cell Physiology 292, C188-C199.

Allen, S., Maden, M., Price, J., 2002. A role for retinoic acid in regulating the regeneration of deer antlers. Dev. Biol. 251, 409-423.

Almudevar, A., Klebanov, L.B., Qiu, X., Salzman, P., Yakovlev, A.Y., 2006. Utility of correlation measures in analysis of gene expression. NeuroRx 3, 384-395.

Alonso, V., Najes, L.M., Provincial, L., Guillén, E., Gil, M., Roncalés, P., Beltrán, J.A., 2012. Influence of dietary fat on pork eating quality. Meat science 92, 366-373.

Amaral, A.J., Ferretti, L., Megens, H.-J., Crooijmans, R., Nie, H., Ramos-Onsins, S.E., Perez-Enciso, M., Schook, L.B., Groenen, M.A., 2011. Genome-wide footprints of pig domestication and selection revealed through massive parallel sequencing of pooled DNA. PloS one 6, e14782.

Ametaj, B.N., Nonnecke, B.J., Franklin, S.T., Horst, R.L., Bidlack, W.R., Stuart, R.L., Beitz, D.C., 2000. Dietary vitamin A modulates the concentrations of RRR-α-tocopherol in plasma lipoproteins from calves fed milk replacer. The Journal of nutrition 130, 629-636.

Anderson, L., Myer, R., Brendemuhl, J., McDowell, L., 1995. The effect of excessive dietary vitamin A on performance and vitamin E status in swine fed diets varying in dietary vitamin E. J. Anim. Sci. 73, 1093-1098.

Andersson, E., Bjorklind, C., Torma, H., Vahlquist, A., 1994. The metabolism of vitamin A to 3,4-didehydroretinol can be demonstrated in human keratinocytes, melanoma cells and HeLa cells, and is correlated to cellular retinoid-binding protein expression. Biochim. Biophys. Acta 1224, 349-354.

Andrés, A.I., Cava, R., Mayoral, A.I., Tejeda, J.F., Morcuende, D., Ruiz, J., 2001. Oxidative stability and fatty acid composition of pig muscles as affected by rearing system, crossbreeding and metabolic type of muscle fibre. Meat Science 59, 39-47.

Andrews, F.N., 1958. 50 Years of Progress in Animal Physiology. J. Anim. Sci. 17, 1064-1078.

Annayev, Y., Adar, S., Chiou, Y.-Y., Lieb, J.D., Sancar, A., Ye, R., 2014. Gene model 129 (Gm129) encodes a novel transcriptional repressor that modulates circadian gene expression. J. Biol. Chem. 289, 5013-5024.

Anwar, K., Iqbal, J., Hussain, M.M., 2007. Mechanisms involved in vitamin E transport by primary enterocytes and in vivo absorption. J. Lipid Res. 48, 2028-2038.

Aparicio Sánchez, G., 1960. Zootecnia especial (etnología compendiada). Imp. Moderna. Córdoba.

Apple, J., Maxwell, C., Brown, D., Friesen, K., Musser, R., Johnson, Z., Armstrong, T., 2004. Effects of dietary lysine and energy density on performance and carcass characteristics of finishing pigs fed ractopamine. J. Anim. Sci. 82, 3277-3287.

Archibald, A.L., Bolund, L., Churcher, C., Fredholm, M., Groenen, M.A., Harlizius, B., Lee, K.-T., Milan, D., Rogers, J., Rothschild, M.F., 2010. Pig genome sequence-analysis and publication strategy. BMC Genomics 11, 438.

Arnett, A., Dikeman, M., Spaeth, C., Johnson, B., Hildabrand, B., 2006. Effects of vitamin A supplementation in young lambs on performance, serum lipid, and carcass adipose tissue attributes. J. Anim. Sci. 84, 111-112.

Arnett, A.M., Dikeman, M.E., Spaeth, C.W., Johnson, B.J., Hildabrand, B., 2007. Effects of vitamin A supplementation in young lambs on performance, serum lipid, and longissimus muscle lipid composition. J. Anim. Sci. 85, 3062-3071.

Asghar, A., Gray, J.I., Booren, A.M., Gomaa, E., Abouzied, M.M., Miller, E.R., Buckley, D.J., 1991. Effects of supranutritional dietary vitamin E levels on subcellular deposition of alpha-tocopherol in the muscle and on pork quality. J. Sci. Food Agric. 57, 31-41.

Ashmore, C., Addis, P., Doerr, L., 1973. Development of muscle fibers in the fetal pig. J. Anim. Sci. 36, 1088-1093.

Association of Official Analytical Chemists, 2005. Official Methods of Analysis., 18 th edition. AOAC,Washington, DC, USA.

Astiz, S., Gonzalez- Bulnes, A., Astiz, I., Barbero, A., Perez- Solana, M., Garcia- Real, I., 2014. Advanced onset of puberty after metformin therapy in swine with thrifty genotype. Experimental physiology 99, 1241-1252.

Ayuso, D., Gonzalez, A., Hernandez, F., Pena, F., Izquierdo, M., 2014. Effect of sex and final fattening on ultrasound and carcass traits in Iberian pigs. Meat Science 96, 562-567.

Ayuso, M., Fernández, A., Isabel, B., Rey, A., Benítez, R., Daza, A., López-Bote, C., Óvilo, C., 2015a. Long term vitamin A restriction improves meat quality parameters and modifies gene expression in Iberian pigs. Journal of Animal Science.

Ayuso, M., Fernandez, A., Nunez, Y., Benitez, R., Isabel, B., Barragán, C., fernandez, A.I., Rey, A.I., Medrano, J.F., Canovas, A., gonzalez-Bulnes, A., López-Bote, C.J., Ovilo, C., 2015b. Comparative analysis of muscle transcriptome between pig genotypes identifies genes and regulatory mechanisms associated to growth, fatness and metabolism. PLoS One. Under reviwe.

Ayuso, M., Ovilo, C., Fernandez, A., Nunez, Y., Isabel, B., Daza, A., Lopez-Bote, C.J., Rey, A.I., 2015c. Effects of dietary vitamin A supplementation or restriction and its timing on retinol and alpha-tocopherol accumulation and gene expression in heavy pigs. Anim. Feed Sci. Technol. 202, 62-74.

Ayuso, M., Óvilo, C., Rodríguez-Bertos, A., Rey, A., Daza, A., Fenández, A., González-Bulnes, A., López-Bote, C., Isabel, B., 2015d. Dietary vitamin A restriction affects adipocyte differentiation and fatty acid composition of intramuscular fat in Iberian pigs. Meat Science 108, 9-16.

Bachar-Dahan, L., Goltzmann, J., Yaniv, A., Gazit, A., 2006. Engrailed-1 Negatively Regulates β-Catenin Transcriptional Activity by Destabilizing β-Catenin via a Glycogen Synthase Kinase-3β–independent Pathway. Mol Biol Cell 17, 2572-2580.

Bahelka, I., Hanusová, E., Peskovicova, D., Demo, P., 2007. The effect of sex and slaughter weight on intramuscular fat content and its relationship to carcass traits of pigs. Czech Journal of Animal Science 52, 122.

Balasubramanyam, A., 2013. The role of the immune system in obesity and insulin resistance. Journal of obesity 2013.

Balmer, J.E., Blomhoff, R., 2002. Gene expression regulation by retinoic acid. J. Lipid Res. 43, 1773-1808.

Balthasar, N., Coppari, R., McMinn, J., Liu, S.M., Lee, C.E., Tang, V., Kenny, C.D., McGovern, R.A., Chua, S.C., Elmquist, J.K., 2004. Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. Neuron 42, 983-991.

Bao, W., Ye, L., Pan, Z., Zhu, J., Du, Z., Zhu, G., Huang, X., Wu, S., 2012. Microarray analysis of differential gene expression in sensitive and resistant pig to Escherichia coli F18. Anim. Genet. 43, 525-534.

Barbero, A., Astiz, S., Lopez-Bote, C.J., Perez-Solana, M.L., Ayuso, M., Garcia-Real, I., Gonzalez-Bulnes, A., 2013. Maternal malnutrition and offspring sex determine juvenile obesity and metabolic disorders in a swine model of leptin resistance. PloS one 8, e78424.

Barbero, A., Garcia-Real, I., Astiz, S., Ayuso, M., Lopez-Bote, C., Gonzalez-Bulnes, A., 2014. Feasibility of MRI and selection of adequate region of interest for longitudinal studies of growth and fatness in swine models of obesity. Diagnostic and interventional imaging 95, 839-847.

Barea, R., Isabel, B., Nieto, R., López-Bote, C., Aguilera, J., 2013. Evolution of the fatty acid profile of subcutaneous back-fat adipose tissue in growing Iberian and Landrace× Large White pigs. Animal 7, 688-698.

Barea, R., Nieto, R., Aguilera, J., 2007. Effects of the dietary protein content and the feeding level on protein and energy metabolism in Iberian pigs growing from 50 to 100 kg body weight.

Barton-Gade, P., Bejerholm, A.C., 1985. Eating quality in pork. Pig farming 33, 56.

Beale, E.G., Harvey, B.J., Forest, C., 2007. PCK1 and PCK2 as candidate diabetes and obesity genes. Cell Biochem Biophys 48, 89-95.

Becker, B., Misfeldt, M., 1993. Evaluation of the mitogen-induced proliferation and cell surface differentiation antigens of lymphocytes from pigs 1 to 30 days of age. J. Anim. Sci. 71, 2073-2078.

Bedo, G., Santisteban, P., Aranda, A., 1989. Retinoic acid regulates growth hormone gene expression.

Bee, G., Gebert, S., Messikommer, R., 2002. Effect of dietary energy supply and fat source on the fatty acid pattern of adipose and lean tissues and lipogenesis in the pig. J. Anim. Sci. 80, 1564-1574.

Bergman, I.-M., Rosengren, J.K., Edman, K., Edfors, I., 2010. European wild boars and domestic pigs display different polymorphic patterns in the Toll-like receptor (TLR) 1, TLR2, and TLR6 genes. Immunogenetics 62, 49-58.

Berkers, C.R., Maddocks, O.D., Cheung, E.C., Mor, I., Vousden, K.H., 2013. Metabolic regulation by p53 family members. Cell metabolism 18, 617-633.

Berry, D.C., Soltanian, H., Noy, N., 2010. Repression of cellular retinoic acid-binding protein II during adipocyte differentiation. J. Biol. Chem. 285, 15324-15332.

Bessa, R., Hughes, R., Jeronimo, E., Moreira, O., Prates, J., Doran, O., 2013. Effect of pig breed and dietary protein level on selected fatty acids and stearoyl-coenzyme A desaturase protein expression in longissimus muscle and subcutaneous fat. J. Anim. Sci. 91, 4540-4546.

Bieri, J.G., Wu, A.-L., Tolliver, T.J., 1981. Reduced intestinal absorption of vitamin E by low dietary levels of retinoic acid in rats. The Journal of nutrition 111, 458-467.

Biesalski, H.K., Frank, J., Beck, S.C., Heinrich, F., Illek, B., Reifen, R., Gollnick, H., Seeliger, M.W., Wissinger, B., Zrenner, E., 1999. Biochemical but not clinical vitamin A deficiency results from mutations in the gene for retinol binding protein. The American journal of clinical nutrition 69, 931-936.

Blair, R., Facon, M., Owen, B., Jacob, J., Bildfell, R., 1996. Tolerance of young pigs for dietary vitamin A and μ-carotene, with special reference to the immune response. Canadian Journal of Animal Science 76, 121-126.

Blakely, S.R., Mitchell, G.V., Jenkins, M.Y., Grundel, E., Whittaker, P., 1991. Canthaxanthin and excess vitamin A alter alpha-tocopherol, carotenoid and iron status in adult rats. The Journal of nutrition 121, 1649-1655.

Blaner, W.S., 1989. Retinol-Binding Protein: The Serum Transport Protein for Vitamin A*. Endocr. Rev. 10, 308-316.

Blaner, W.S., and J. A. Olson, 1994. The retinoids: Biology, chemistry and medicine (second edition) Edited by M B Sporn, A B Roberts and the late D S Goodman. Raven Press, NY. Biochemical Education 22, 5-178.

Bligh, E.G., Dyer, W.J., 1959. A Rapid Method of Total Lipid Extraction and Purification. Can J Biochem Phys 37, 911-917.

Blüher, M., Michael, M.D., Peroni, O.D., Ueki, K., Carter, N., Kahn, B.B., Kahn, C.R., 2002. Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. Dev. Cell 3, 25-38.

Bonet, M., Ribot, J., Felipe, F., Palou, A., 2003. Vitamin A and the regulation of fat reserves. Cellular and Molecular Life Sciences CMLS 60, 1311-1321.

Bonet, M.L., Puigserver, P., Serra, F., Ribot, J., Vazquez, F., Pico, C., Palou, A., 1997. Retinoic acid modulates retinoid X receptor alpha and retinoic acid receptor alpha levels of cultured brown adipocytes. FEBS Lett. 406, 196-200.

Bonnefond, A., Raimondo, A., Stutzmann, F., Ghoussaini, M., Ramachandrappa, S., Bersten, D.C., Durand, E., Vatin, V., Balkau, B., Lantieri, O., Raverdy, V., Pattou, F., Van Hul, W., Van Gaal, L., Peet, D.J., Weill, J., Miller, J.L., Horber, F., Goldstone, A.P., Driscoll, D.J., Bruning, J.B., Meyre, D., Whitelaw, M.L., Froguel, P., 2013. Loss-of-function mutations in SIM1 contribute to obesity and Prader-Willi-like features. J. Clin. Invest. 123, 3037-3041.

Boone, C., Gregoire, F., Remacle, C., 2000. Culture of porcine stromal-vascular cells in serum-free medium: differential action of various hormonal agents on adipose conversion. J. Anim. Sci. 78, 885-895.

Borselli, C., Storrie, H., Benesch-Lee, F., Shvartsman, D., Cezar, C., Lichtman, J.W., Vandenburgh, H.H., Mooney, D.J., 2010. Functional muscle regeneration with combined delivery of angiogenesis and myogenesis factors. Proceedings of the National Academy of Sciences 107, 3287-3292.

Bosch, L., Tor, M., Reixach, J., Estany, J., 2012. Age-related changes in intramuscular and subcutaneous fat content and fatty acid composition in growing pigs using longitudinal data. Meat Science 91, 358-363.

Bost, F., Caron, L., Marchetti, I., Dani, C., Le Marchand-Brustel, Y., Binétruy, B., 2002. Retinoic acid activation of the ERK pathway is required for embryonic stem cell commitment into the adipocyte lineage. Biochem. J 361, 621-627.

Bourin, P., Bunnell, B.A., Casteilla, L., Dominici, M., Katz, A.J., March, K.L., Redl, H., Rubin, J.P., Yoshimura, K., Gimble, J.M., 2013. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). Cytotherapy 15, 641-648.

Boyle, K.B., Hadaschik, D., Virtue, S., Cawthorn, W.P., Ridley, S.H., O'Rahilly, S., Siddle, K., 2009. The transcription factors Egr1 and Egr2 have opposing influences on adipocyte differentiation. Cell Death & Differentiation 16, 782-789.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Bradshaw, R.A., Noyes, B.E., 1975. L-3-hydroxyacyl coenzyme A dehydrogenase from pig heart muscle, In: John, M.L. (Ed.), Methods Enzymol., Academic Press, pp. 122-128.

Brandebourg, T.D., Hu, C.Y., 2005. Regulation of differentiating pig preadipocytes by retinoic acid. J. Anim. Sci. 83, 98-107.

Bredt, D.S., 2003. Nitric oxide signaling specificity—the heart of the problem. J. Cell Sci. 116, 9-15.

Briata, P., Lin, W.-J., Giovarelli, M., Pasero, M., Chou, C.-F., Trabucchi, M., Rosenfeld, M.G., Chen, C.-Y., Gherzi, R., 2012. PI3K/AKT signaling determines a dynamic switch between distinct KSRP functions favoring skeletal myogenesis. Cell Death & Differentiation 19, 478-487.

Brief, S., Chew, B., 1985. Effects of Vitamin A and-Carotene on Reproductive Performance in Gilts. J. Anim. Sci. 60, 998-1004.

Brun, P.J., Yang, K.J.Z., Lee, S.A., Yuen, J.J., Blaner, W.S., 2013. Retinoids: Potent regulators of metabolism. BioFactors 39, 151-163.

Buckley, D., Morrissey, P., Gray, J., 1995. Influence of dietary vitamin E on the oxidative stability and quality of pig meat. J. Anim. Sci. 73, 3122-3130.

Burdge, G.C., Slater-Jefferies, J., Torrens, C., Phillips, E.S., Hanson, M.A., Lillycrop, K.A., 2007. Dietary protein restriction of pregnant rats in the F 0 generation induces altered methylation of hepatic gene promoters in the adult male offspring in the F 1 and F 2 generations. British Journal of Nutrition 97, 435-439.

Butler, A.A., Roith, D.L., 2001. CONTROL OF GROWTH BY THE SOMATROPIC AXIS: Growth Hormone and the Insulin-Like Growth Factors Have Related and Independent Roles 1. Annu. Rev. Physiol. 63, 141-164.

Cagnazzo, M., Te Pas, M., Priem, J., De Wit, A., Pool, M., Davoli, R., Russo, V., 2006. Comparison of prenatal muscle tissue expression profiles of two pig breeds differing in muscle characteristics. J. Anim. Sci. 84, 1-10.

Čandek-Potokar, M., Žlender, B., Bonneau, M., 1998. Effects of breed and slaughter weight on longissimus muscle biochemical traits and sensory quality in pigs, Ann. Zootech., EDP Sciences, pp. 3-16.

Cánovas, A., Rincón, G., Bevilacqua, C., Islas-Trejo, A., Brenaut, P., Hovey, R.C., Boutinaud, M., Morgenthaler, C., VanKlompenberg, M.K., Martin, P., 2014. Comparison of five different RNA sources to examine the lactating bovine mammary gland transcriptome using RNA-Sequencing. Scientific reports 4.

Cantile, M., Procino, A., D'Armiento, M., Cindolo, L., Cillo, C., 2003. HOX gene network is involved in the transcriptional regulation of in vivo human adipogenesis. Journal of Cellular Physiology 194, 225-236.

Cao, Z., Umek, R.M., McKnight, S.L., 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev. 5, 1538-1552.

Carrapiso, A.I., Bonilla, F., García, C., 2003. Effect of crossbreeding and rearing system on
sensory characteristics of Iberian ham. Meat science 65, 623-629.

Carrapiso, A.I., García, C., 2005. Instrumental colour of Iberian ham subcutaneous fat and lean (biceps femoris): influence of crossbreeding and rearing system. Meat Science 71, 284-290.

Carrasco, V., Canfrán, S., Rodríguez-Franco, F., Benito, A., Sáinz, A., Rodríguez-Bertos, A., 2011. Canine Gastric Carcinoma Immunohistochemical Expression of Cell Cycle Proteins (p53, p21, and p16) and Heat Shock Proteins (Hsp27 and Hsp70). Veterinary Pathology Online 48, 322-329.

Casas- Díaz, E., Closa- Sebastià, F., Marco, I., Lavín, S., Bach- Raich, E., Cuenca, R., 2015. Hematologic and biochemical reference intervals for Wild Boar (Sus scrofa) captured by cage trap. Veterinary Clinical Pathology.

Castell, A., Cliplef, R., Poste-Flynn, L., Butler, G., 1994. Performance, carcass and pork characteristics of castrates and gilts self-fed diets differing in protein content and lysine: energy ratio. Canadian Journal of Animal Science 74, 519-528.

Castellano, R., Perruchot, M.-H., Conde-Aguilera, J.A., van Milgen, J., Collin, A., Tesseraud, S., Mercier, Y., Gondret, F., 2015. A Methionine Deficient Diet Enhances Adipose Tissue Lipid Metabolism and Alters Anti-Oxidant Pathways in Young Growing Pigs. Plos One 10.

Castro-Muñozledo, F., Marsch-Moreno, M., Beltràn-Langarica, A., Kuri-Harcuch, W., 1987. Commitment of adipocyte differentiation in 3T3 cells is inhibited by retinoic acid, and the expression of lipogenic enzymes is modulated through cytoskeleton stabilization. Differentiation 36, 211-219.

Cava, R., Ruiz, J., López-Bote, C., Martín, L., García, C., Ventanas, J., Antequera, T., 1997. Influence of finishing diet on fatty acid profiles of intramuscular lipids, triglycerides and phospholipids in muscles of the Iberian pig. Meat Science 45, 263-270.

Cava, R., Ruiz, J., Ventanas, J., Antequera, T., 1999. Oxidative and lipolytic changes during ripening of Iberian hams as affected by feeding regime: extensive feeding and alpha-tocopheryl acetate supplementation. Meat Science 52, 165-172.

Cawthorn, W.P., Scheller, E.L., MacDougald, O.A., 2012. Adipose tissue stem cells meet preadipocyte commitment: going back to the future. J. Lipid Res. 53, 227-246.

Čepica, S., Ovilo, C., Masopust, M., Knoll, A., Fernandez, A., Lopez, A., Rohrer, G., Nonneman, D., 2012. Four genes located on a SSC2 meat quality QTL region are associated with different meat quality traits in Landrace× Chinese- European crossbred population. Anim. Genet. 43, 333-336.

Chambon, P., 1996. A decade of molecular biology of retinoic acid receptors. The FASEB Journal 10, 940-954.

Chang, K., Da Costa, N., Blackley, R., Southwood, O., Evans, G., Plastow, G., Wood, J., Richardson, R., 2003. Relationships of myosin heavy chain fibre types to meat quality traits in traditional and modern pigs. Meat Science 64, 93-103.

Chawla, A., Repa, J.J., Evans, R.M., Mangelsdorf, D.J., 2001. Nuclear receptors and lipid physiology: opening the X-files. Science 294, 1866-1870.

Chen, G., Wang, C., Shi, T., 2011. Overview of available methods for diverse RNA-Seq data analyses. Science China Life Sciences 54, 1121-1128.

Chen, S.N., Czernuszewicz, G., Tan, Y., Lombardi, R., Jin, J., Willerson, J.T., Marian, A.J., 2012. Human molecular genetic and functional studies identify TRIM63, encoding Muscle RING Finger Protein 1, as a novel gene for human hypertrophic cardiomyopathy. Circul. Res. 111, 907-919.

Chen, Z., Torrens, J.I., Anand, A., Spiegelman, B.M., Friedman, J.M., 2005. Krox20 stimulates adipogenesis via CEBPB-dependent and-independent mechanisms. Cell Metabolism 1, 93-106.

Ching, S., Mahan, D., Wiseman, T., Fastinger, N., 2002. Evaluating the antioxidant status of weanling pigs fed dietary vitamins A and E. J. Anim. Sci. 80, 2396-2401.

Cho, O.H., Mallappa, C., Hernández- Hernández, J.M., Rivera- Pérez, J.A., Imbalzano, A.N., 2015. Contrasting roles for MyoD in organizing myogenic promoter structures during embryonic skeletal muscle development. Dev. Dyn. 244, 43-55.

Choy, L., Derynck, R., 2003. Transforming growth factor-β inhibits adipocyte differentiation by Smad3 interacting with CCAAT/enhancer-binding protein (C/EBP) and repressing C/EBP transactivation function. J. Biol. Chem. 278, 9609-9619.

Cilla, I., Altarriba, J., Guerrero, L., Gispert, M., Martínez, L., Moreno, C., Beltrán, J.A., Guàrdia, M.D., Diestre, A., Arnau, J., 2006. Effect of different Duroc line sires on carcass composition, meat quality and dry-cured ham acceptability. Meat Science 72, 252-260.

Cisneros, F., Ellis, M., Baker, D., Easter, R., McKeith, F., 1996a. The influence of short-term feeding of amino acid-deficient diets and high dietary leucine levels on the intramuscular fat content of pig muscle. Animal Science 63, 517-522.

Cisneros, F., Ellis, M., McKeith, F., McCaw, J., Fernando, R., 1996b. Influence of slaughter weight on growth and carcass characteristics, commercial cutting and curing yields, and meat quality of barrows and gilts from two genotypes. J. Anim. Sci. 74, 925-933.

Clawitter, J., Trout, W.E., Burke, M.G., Araghi, S., Roberts, R.M., 1990. A novel family of progesterone-induced, retinol-binding proteins from uterine secretions of the pig. J. Biol. Chem. 265, 3248-3255.

Coffey, M., Britt, J., 1993. Enhancement of sow reproductive performance by beta-carotene or vitamin A. J. Anim. Sci. 71, 1198-1202.

Coffey, M., Seerley, R., Funderburke, D., McCampbell, H., 1982. Effect of heat increment and level of dietary energy and environmental temperature on the performance of growing-finishing swine. J. Anim. Sci. 54, 95-105.

Coleman, R.A., Haynes, E.B., 1984. Microsomal and lysosomal enzymes of triacylglycerol metabolism in rat placenta. Biochem. J 217, 391-397.

Cordero, G., Isabel, B., Menoyo, D., Daza, A., Morales, J., Piñeiro, C., López-Bote, C., 2010. Dietary CLA alters intramuscular fat and fatty acid composition of pig skeletal muscle and subcutaneous adipose tissue. Meat Science 85, 235-239.

Corella, D., Ordovas, J.M., 2009. Nutrigenomics in cardiovascular medicine. Circulation: Cardiovascular Genetics 2, 637-651.

Corominas, J., Ramayo-Caldas, Y., Puig-Oliveras, A., Estelle, J., Castello, A., Alves, E., Pena, R.N., Ballester, M., Folch, J.M., 2013a. Analysis of porcine adipose tissue transcriptome reveals differences in de novo fatty acid synthesis in pigs with divergent muscle fatty acid composition. BMC Genomics 14, 843.

Corominas, J., Ramayo-Caldas, Y., Puig-Oliveras, A., Pérez-Montarelo, D., Noguera, J.L., Folch, J.M., Ballester, M., 2013b. Polymorphism in the ELOVL6 gene is associated with a major QTL effect on fatty acid composition in pigs. PLoS One 8, e53687.

Correa, J., Faucitano, L., Laforest, J., Rivest, J., Marcoux, M., Gariépy, C., 2006. Effects of slaughter weight on carcass composition and meat quality in pigs of two different growth rates. Meat Science 72, 91-99.

Coumailleau, P., Duprez, D., 2009. Sim1 and Sim2 expression during chick and mouse limb development. Int. J. Dev. Biol. 53, 149-157.

Cromwell, G., Hays, V., Clark, T., 1978. Effect of Copper Sulfate, Copper Sulfide and Sodium Sulfide on Performance and Copper Stores of Pigs. J. Anim. Sci. 46, 692-698.

Cronan, J.E., Waldrop, G.L., 2002. Multi-subunit acetyl-CoA carboxylases. Prog Lipid Res 41, 407-435.

D'Souza, D.N., Pethick, D.W., Dunshea, F.R., Pluske, J.R., Mullan, B.P., 2003. Nutritional manipulation increases intramuscular fat levels in the Longissimus muscle of female finisher pigs. Aust. J. Agric. Res. 54, 745-749.

D'Ambrosio, D.N., Clugston, R.D., Blaner, W.S., 2011. Vitamin A metabolism: an update. Nutrients 3, 63-103.

D'Andrea, M., Dal Monego, S., Pallavicini, A., Modonut, M., Dreos, R., Stefanon, B., Pilla, F., 2011. Muscle transcriptome profiling in divergent phenotype swine breeds during growth using microarray and RT- PCR tools. Anim. Genet. 42, 501-509.

Damon, M., Louveau, I., Lefaucheur, L., Lebret, B., Vincent, A., Leroy, P., Sanchez, M.P., Herpin, P., Gondret, F., 2006. Number of intramuscular adipocytes and fatty acid binding protein-4 content are significant indicators of intramuscular fat level in crossbred Large White x Duroc pigs. J. Anim. Sci. 84, 1083-1092.

Damon, M., Wyszynska-Koko, J., Vincent, A., Herault, F., Lebret, B., 2012. Comparison of muscle transcriptome between pigs with divergent meat quality phenotypes identifies genes related to muscle metabolism and structure. PLoS One 7, e33763.

Dani, C., Smith, A., Dessolin, S., Leroy, P., Staccini, L., Villageois, P., Darimont, C., Ailhaud, G., 1997. Differentiation of embryonic stem cells into adipocytes in vitro. J. Cell Sci. 110, 1279-1285.

Daniel, Z.C.T.R., Salter, A.M., Buttery, P.J., 2004. Vitamin A regulation of stearoyl-CoA desaturase mRNA levels and fatty acid composition in sheep tissues. Animal Science 78, 237-243.

Darlington, G.J., Ross, S.E., MacDougald, O.A., 1998. The role of C/EBP genes in adipocyte differentiation. J. Biol. Chem. 273, 30057-30060.

Darnell, J.E., 1997. STATs and gene regulation. Science 277, 1630-1635.

Darroch, C., 2001. Vitamin A in swine nutrition, In: Lewis, A., Southern, L. (Eds.), Swine nutrition. 2nd edition. CRC Press, Boca Raton, FL, USA, pp. 263-280.

Daza, A., Lopez-Bote, C.J., Olivares, A., Menoyo, D., Ruiz, J., 2007. Age at the beginning of the fattening period of Iberian pigs under free-range conditions affects growth, carcass characteristics and the fatty acid profile of lipids. Anim. Feed Sci. Technol. 139, 81-91.

Daza, A., Rey, A., Ruiz, J., Lopez-Bote, C., 2005. Effects of feeding in free-range conditions or in confinement with different dietary MUFA/PUFA ratios and α-tocopheryl acetate, on antioxidants accumulation and oxidative stability in Iberian pigs. Meat Science 69, 151-163.

De Blas, C., Gasa, J., Mateos, G.G., 2013. Fundación Española Desarrollo Nutrición Animal, Madrid, Spain.

De la Llata, M., Dritz, S., Tokach, M., Goodband, R., Nelssen, J., Loughin, T., 2001. Effects of dietary fat on growth performance and carcass characteristics of growing-finishing pigs reared in a commercial environment. J. Anim. Sci. 79, 2643-2650.

de Lira, L.Q., Lima, M.S.R., de Medeiros, J.M.S., da Silva, I.F., Dimenstein, R., 2013. Correlation of vitamin A nutritional status on alpha- tocopherol in the colostrum of lactating women. Maternal & child nutrition 9, 31-40.

de Sousa, U.L.J., Koss, M.D., Fillies, M., Gahl, A., Scheeder, M.R., Cardoso, M.C., Leonhardt, H., Geary, N., Langhans, W., Leonhardt, M., 2005. CPT1α over-expression increases long-chain fatty acid oxidation and reduces cell viability with incremental palmitic acid concentration in 293T cells. Biochem. Biophys. Res. Commun. 338, 757-761.

DeBerardinis, R.J., Mancuso, A., Daikhin, E., Nissim, I., Yudkoff, M., Wehrli, S., Thompson, C.B., 2007. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. Proceedings of the National Academy of Sciences 104, 19345-19350.

Deiuliis, J.A., Li, B., Lyvers-Peffer, P.A., Moeller, S.J., Lee, K., 2006. Alternative splicing of delta-like 1 homolog (< i> DLK1</i>) in the pig and human. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 145, 50-59.

Demmerle, J., Koch, A.J., Holaska, J.M., 2013. Emerin and histone deacetylase 3 (HDAC3) cooperatively regulate expression and nuclear positions of MyoD, Myf5, and Pax7 genes during myogenesis. Chromosome Res 21, 765-779.

DeVol, D., McKeith, F., Bechtel, P.J., Novakofski, J., Shanks, R., Carr, T., 1988. Variation in composition and palatability traits and relationships between muscle characteristics and palatability in a random sample of pork carcasses. J. Anim. Sci. 66, 385-395.

Dieguez, E., 1992. Historia, evolución y situación actual del cerdo ibérico. Cerdo Ibérico, la naturaleza, la dehesa. (Ed. Ministerio de Agricultura, Pesca y Alimentación), Ministerio de Agricultura, Pesca y Alimentación, Madrid.

Dikeman, M.E., 2007. Effects of metabolic modifiers on carcass traits and meat quality. Meat Science 77, 121-135.

Diplock, A., 1983. The role of vitamin E in biological membranes, Biology of vitamin E, Pitman Books London, pp. 45-55.

Dirinck, P., De Winne, A., Casteels, M., Frigg, M., 1996. Studies on vitamin E and meat quality. 1. Effect of feeding high vitamin E levels on time-related pork quality. J. Agric. Food Chem. 44, 65-68.

Dolle, P., Fraulob, V., Kastner, P., Chambon, P., 1994. Developmental expression of murine retinoid X receptor (RXR) genes. Mech Dev 45, 91-104.

Domínguez, R., Martínez, S., Carballo, J., Franco, I., 2014. Fatty acid profile and cholesterol and retinol contents in different locations of Celta pig breed. Grasas Aceites 65, e036.

Dong, D., Ruuska, S.E., Levinthal, D.J., Noy, N., 1999. Distinct roles for cellular retinoic acid-binding proteins I and II in regulating signaling by retinoic acid. J. Biol. Chem. 274, 23695-23698.

Duester, G., 2000. Families of retinoid dehydrogenases regulating vitamin A function. Eur. J. Biochem. 267, 4315-4324.

Duester, G., Mic, F.A., Molotkov, A., 2003. Cytosolic retinoid dehydrogenases govern ubiquitous metabolism of retinol to retinaldehyde followed by tissue-specific metabolism to retinoic acid. Chem.-Biol. Interact. 143, 201-210.

Dunn, J.R., Reed, J., Du Plessis, D., Shaw, E., Reeves, P., Gee, A., Warnke, P., Walker, C., 2006. Expression of ADAMTS-8, a secreted protease with antiangiogenic properties, is downregulated in brain tumours. Br. J. Cancer 94, 1186-1193.

Dunshea, F., D'souza, D., Pethick, D., Harper, G., Warner, R., 2005. Effects of dietary factors and other metabolic modifiers on quality and nutritional value of meat. Meat Science 71, 8-38.

Dunshea, F.R., D'Sousa, D.N., 2003. Fat deposition in the pig, In: Paterson, J.A. (Ed.), MAnipulating pig production, Australian Pig Science Association, Werribee, Australia, pp. 127-150.

Durgan, D.J., Smith, J.K., Hotze, M.A., Egbejimi, O., Cuthbert, K.D., Zaha, V.G., Dyck, J.R., Abel, E.D., Young, M.E., 2006. Distinct transcriptional regulation of long-chain acyl-CoA synthetase isoforms and cytosolic thioesterase 1 in the rodent heart by fatty acids and insulin. Am. J. Physiol. Heart Circ. Physiol. 290, H2480-2497.

Ebert, S.M., Monteys, A.M., Fox, D.K., Bongers, K.S., Shields, B.E., Malmberg, S.E., Davidson, B.L., Suneja, M., Adams, C.M., 2010. The transcription factor ATF4 promotes skeletal myofiber atrophy during fasting. Mol. Endocrinol. 24, 790-799.

Edmondson, D.G., Cheng, T., Cserjesi, P., Chakraborty, T., Olson, E.N., 1992. Analysis of the myogenin promoter reveals an indirect pathway for positive autoregulation mediated by the muscle-specific enhancer factor MEF-2. Mol. Cell. Biol. 12, 3665-3677.

Edwards, S., 2005. Product quality attributes associated with outdoor pig production. Livestock Production Science 94, 5-14.

Eicher, S., Morrill, J., Velazco, J., 1997. Bioavailability of α-tocopherol fed with retinol and relative bioavailability of d-α-tocopherol or dl-α-tocopherol acetate. J. Dairy Sci. 80, 393-399.

Elias, C.F., Aschkenasi, C., Lee, C., Kelly, J., Ahima, R.S., Bjorbæk, C., Flier, J.S., Saper, C.B., Elmquist, J.K., 1999. Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. Neuron 23, 775-786.

Ellis, M., Webb, A., Avery, P., Brown, I., 1996. The influence of terminal sire genotype, sex, slaughter weight, feeding regime and slaughter-house on growth performance and carcass and meat quality in pigs and on the organoleptic properties of fresh pork. Animal Science 62, 521-530.

Esteve-Codina, A., Kofler, R., Palmieri, N., Bussotti, G., Notredame, C., Pérez-Enciso, M., 2011. Exploring the gonad transcriptome of two extreme male pigs with RNA-seq. BMC Genomics 12, 552.

Exley, M.A., Hand, L., O'Shea, D., Lynch, L., 2014. Interplay between the immune system and adipose tissue in obesity. J. Endocrinol. 223, R41-R48.

Fan, B., Lkhagvadorj, S., Cai, W., Young, J., Smith, R., Dekkers, J., Huff-Lonergan, E., Lonergan, S., Rothschild, M., 2010. Identification of genetic markers associated with residual feed intake and meat quality traits in the pig. Meat Science 84, 645-650.

Farmer, S.R., 2006. Transcriptional control of adipocyte formation. Cell metabolism 4, 263-273.

Faust, I.M., Johnson, P.R., Stern, J.S., Hirsch, J., 1978. Diet-induced adipocyte number increase in adult rats: a new model of obesity. Am J Physiol-Endoc M 235, E279.

Fernandez, I., Darias, M., Andree, K.B., Mazurais, D., Zambonino-Infante, J.L., Gisbert, E., 2011. Coordinated gene expression during gilthead sea bream skeletogenesis and its disruption by nutritional hypervitaminosis A. BMC Dev. Biol. 11, 7.

Fernandez, X., Monin, G., Talmant, A., Mourot, J., Lebret, B., 1999. Influence of intramuscular fat content on the quality of pig meat—2. Consumer acceptability of m. longissimus lumborum. Meat Science 53, 67-72.

Fernandez-Figares, I., Lachica, M., Nieto, R., Rivera-Ferre, M., Aguilera, J., 2007. Serum profile of metabolites and hormones in obese (Iberian) and lean (Landrace) growing gilts fed balanced or lysine deficient diets. Livest Sci 110, 73-81.

Fernandez-Rodriguez, A., Munoz, M., Fernandez, A., Pena, R.N., Tomas, A., Noguera, J.L., Ovilo, C., Fernandez, A.I., 2011. Differential gene expression in ovaries of pregnant pigs with high and low prolificacy levels and identification of candidate genes for litter size. Biol. Reprod. 84, 299-307.

Ferraz, A.L.J., Ojeda, A., Lopez-Bejar, M., Fernandes, L.T., Castello, A., Folch, J.M., Perez-Enciso, M., 2008. Transcriptome architecture across tissues in the pig. BMC Genomics 9.

Ferrini, G., Manzanilla, E.G., Menoyo, D., Esteve-Garcia, E., Baucells, M.D., Barroeta, A.C., 2010. Effects of dietary n-3 fatty acids in fat metabolism and thyroid hormone levels when compared to dietary saturated fatty acids in chickens. Livest Sci 131, 287-291.

Firdous, S., 2014. Correlation of CRP, Fasting Serum Triglycerides and Obesity as Cardiovascular Risk Factors. Jcpsp-Journal of the College of Physicians and Surgeons Pakistan 24, 308-313.

Fischer, D., Laiho, A., Gyenesei, A., Sironen, A., 2015. Identification of Reproduction Related Gene Polymorphisms Using Whole Transcriptome Sequencing in the Large White Pig Population. G3: Genes| Genomes| Genetics, g3. 115.018382.

Flachowsky, G., Schulz, E., Kratz, R., Glodek, P., 2008. Effects of different dietary fat sources on the fatty acid profile of backfat and intramuscular fat of pigs of various sire breeds. Journal of Animal and Feed Sciences 17, 363-371.

Florez, J.C., Jablonski, K.A., Bayley, N., Pollin, T.I., de Bakker, P.I., Shuldiner, A.R., Knowler, W.C., Nathan, D.M., Altshuler, D., 2006. TCF7L2 polymorphisms and progression to diabetes in the Diabetes Prevention Program. New Engl. J. Med. 355, 241-250.

Fonken, L.K., Nelson, R.J., 2014. The effects of light at night on circadian clocks and metabolism. Endocr. Rev. 35, 648-670.

Fraga, M., Villamide, M., 2000. The composition of vitamin supplements in Spanish pig diets. Pig News and Information 21, 67N-72N.

Francesc, R., Joan, V., Elayne, H., Marta, G., Francesc, V., 2014. FGF21 expression and release in muscle cells: involvement of MyoD and regulation by mitochondria-driven signalling. Biochem. J. 463, 191-199.

Franks, P.W., Christophi, C.A., Jablonski, K.A., Billings, L.K., Delahanty, L.M., Horton, E.S., Knowler, W.C., Florez, J.C., Group, D.P.P.R., 2014. Common variation at PPARGC1A/B and change in body composition and metabolic traits following preventive interventions: the Diabetes Prevention Program. Diabetologia 57, 485-490.

Frateschi, S., Keppner, A., Malsure, S., Iwaszkiewicz, J., Sergi, C., Merillat, A.-M., Fowler-Jaeger, N., Randrianarison, N., Planès, C., Hummler, E., 2012. Mutations of the serine protease CAP1/Prss8 lead to reduced embryonic viability, skin defects, and decreased ENaC activity. The American journal of pathology 181, 605-615.

Frey, S.K., Vogel, S., 2011. Vitamin A Metabolism and Adipose Tissue Biology. Nutrients 3, 27-39.

Freytag, S.O., Paielli, D.L., Gilbert, J.D., 1994. Ectopic expression of the CCAAT/enhancer-binding protein alpha promotes the adipogenic program in a variety of mouse fibroblastic cells. Genes Dev. 8, 1654-1663.

Fu, Z., Noguchi, T., Kato, H., 2001. Vitamin A deficiency reduces insulin-like growth factor (IGF)-I gene expression and increases IGF-I receptor and insulin receptor gene expression in tissues of Japanese quail (Coturnix coturnix japonica). J. Nutr. 131, 1189-1194.

Fuentes, V., Ventanas, S., Ventanas, J., Estevez, M., 2014. The genetic background affects composition, oxidative stability and quality traits of Iberian dry-cured hams: Purebred Iberian versus reciprocal Iberian x Duroc crossbred pigs. Meat Science 96, 737-743.

Garcia de Herreros, A., Birnbaum, M.J., 1989. The regulation by insulin of glucose transporter gene expression in 3T3 adipocytes. J. Biol. Chem. 264, 9885-9890.

Garcia, L.A., Ferrini, M.G., Norris, K.C., Artaza, J.N., 2013. 1, 25 (OH) 2 vitamin D 3 enhances myogenic differentiation by modulating the expression of key angiogenic growth factors and angiogenic

inhibitors in C 2 C 12 skeletal muscle cells. The Journal of steroid biochemistry and molecular biology 133, 1-11.
Gatlin, L.A., See, M., Hansen, J., Sutton, D., Odle, J., 2002. The effects of dietary fat sources, levels, and feeding intervals on pork fatty acid composition. J. Anim. Sci. 80, 1606-1615.
Gerbens, F., 2004. 16 Genetic Control of Intramuscular Fat Accretion, In: te Pas, M.F., Everts, M.E., Haagsman, H.P. (Eds.), Muscle development of livestock animals: Physiology, genetics and meat quality, CABI, p. 343.
Gerfault, V., Louveau, I., Mourot, J., 1999. The effect of GH and IGF-I on preadipocytes from Large White and Meishan pigs in primary culture. Gen. Comp. Endocrinol. 114, 396-404.
Ghosh, M., Sodhi, S., Song, K.D., Kim, J., Mongre, R., Sharma, N., Singh, N., Kim, S., Lee, H., Jeong, D., 2015. Evaluation of body growth and immunity- related differentially expressed genes through deep RNA sequencing in the piglets of Jeju native pig and Berkshire. Anim. Genet. 46, 255-264.
Giambanelli, E., Ferioli, F., Koçaoglu, B., Jorjadze, M., Alexieva, I., Darbinyan, N., D'Antuono, L.F., 2013. A comparative study of bioactive compounds in primitive wheat populations from Italy, Turkey, Georgia, Bulgaria and Armenia. J. Sci. Food Agric. 93, 3490-3501.
Goddard, M.E., Hayes, B.J., 2009. Mapping genes for complex traits in domestic animals and their use in breeding programmes. Nature Reviews Genetics 10, 381-391.
Goerl, K., Eilert, S., Mandigo, R., Chen, H., Miller, P., 1995. Pork characteristics as affected by two populations of swine and six crude protein levels. J. Anim. Sci. 73, 3621-3626.
Goldberg, A.D., Allis, C.D., Bernstein, E., 2007. Epigenetics: a landscape takes shape. Cell 128, 635-638.
Gondret, F., Guitton, N., Guillerm-Regost, C., Louveau, I., 2008. Regional differences in porcine adipocytes isolated from skeletal muscle and adipose tissues as identified by a proteomic approach. J. Anim. Sci. 86, 2115-2125.
Gondret, F., Lebret, B., 2002. Feeding intensity and dietary protein level affect adipocyte cellularity and lipogenic capacity of muscle homogenates in growing pigs, without modification of the expression of sterol regulatory element binding protein's. J. Anim. Sci. 80, 3184-3193.
Gondret, F., Perruchot, M., Tacher, S., Berard, J., Bee, G., 2011. Differential gene expressions in subcutaneous adipose tissue pointed to a delayed adipocytic differentiation in small pig fetuses compared to their heavier siblings. Differentiation 81, 253-260.
Gonzalez-Bulnes, A., Ovilo, C., Lopez-Bote, C.J., Astiz, S., Ayuso, M., Perez-Solana, M., Sanchez-Sanchez, R., Torres-Rovira, L., 2012a. Gender-specific early postnatal catch-up growth after intrauterine growth retardation by food restriction in swine with obesity/leptin resistance. Reproduction 144, 269-278.
Gonzalez-Bulnes, A., Torres-Rovira, L., Ovilo, C., Astiz, S., Gomez-Izquierdo, E., Gonzalez-Añover, P., Pallares, P., Perez-Solana, M., Sanchez-Sanchez, R., 2012b. Reproductive, endocrine and metabolic feto-maternal features and placental gene expression in a swine breed with obesity/leptin resistance. Gen. Comp. Endocrinol. 176, 94-101.
Gonzalez- Añover, P., Encinas, T., Gomez- Izquierdo, E., Sanz, E., Letelier, C., Torres- Rovira, L., Pallares, P., Sanchez- Sanchez, R., Gonzalez- Bulnes, A., 2010. Advanced onset of puberty in gilts of thrifty genotype (Iberian pig). Reproduction in domestic animals 45, 1003-1007.
Goodman, D.S., 1984a. Vitamin A and retinoids in health and disease. The New England journal of medicine 310, 1023-1031.
Goodman, D.S., 1984b. The Retinoids. Elsevier Science, Academic Press, Orlando, FL.
Goodman, G.E., Metch, B.J., Omenn, G.S., 1994. The effect of long-term beta-carotene and vitamin A administration on serum concentrations of alpha-tocopherol. Cancer Epidem Biomar 3, 429-432.
Gorocica-Buenfil, M., Fluharty, F., Bohn, T., Tirabasso, P., Lowe, G., Loerch, S., 2007a. Effect of low vitamin A diets with high-moisture- or dry-corn on marbling and adipose tissue fatty acid composition of beef steers. J. Anim. Sci. 85, 3355-3366.
Gorocica-Buenfil, M., Fluharty, F., Loerch, S., 2008. Effect of vitamin A restriction on carcass characteristics and immune status of beef steers. J. Anim. Sci. 86, 1609-1616.
Gorocica-Buenfil, M., Fluharty, F., Reynolds, C., Loerch, S., 2007b. Effect of dietary vitamin A concentration and roasted soybean inclusion on marbling, adipose cellularity, and fatty acid composition of beef. J. Anim. Sci. 85, 2230-2242.
Gorocica-Buenfil, M., Fluharty, F., Reynolds, C.. Loerch, S., 2007c. Effect of dietary vitamin A restriction on marbling and conjugated linoleic acid content in Holstein steers. J. Anim. Sci. 85, 2243-2255.

Granucci, F., Petralia, F., Urbano, M., Citterio, S., Di Tota, F., Santambrogio, L., Ricciardi-Castagnoli, P., 2003. The scavenger receptor MARCO mediates cytoskeleton rearrangements in dendritic cells and microglia. Blood 102, 2940-2947.

Gregoire, F.M., Smas, C.M., Sul, H.S., 1998. Understanding adipocyte differentiation. Physiol. Rev. 78, 783-809.

Groenen, M.A., Archibald, A.L., Uenishi, H., Tuggle, C.K., Takeuchi, Y., Rothschild, M.F., Rogel-Gaillard, C., Park, C., Milan, D., Megens, H.-J., 2012. Analyses of pig genomes provide insight into porcine demography and evolution. Nature 491, 393-398.

Groff, J., Gropper, S., Hunt, S., 1995. The water soluble vitamins. Advanced nutrition and human metabolism 3, 289-297.

Grun, F., Blumberg, B., 2006. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. Endocrinology 147, s50-s55.

Guo, Y., Jin, L., Wang, F., He, M., Liu, R., Li, M., Shuai, S., 2014. Dynamic changes in genes related to glucose uptake and utilization during pig skeletal and cardiac muscle development. Biosci., Biotechnol., Biochem. 78, 1159-1166.

Gupta, D., Leahy, A.A., Monga, N., Peshavaria, M., Jetton, T.L., Leahy, J.L., 2013. Peroxisome Proliferator-activated Receptor G (PPARG) and Its Target Genes Are Downstream Effectors of FoxO1 Protein in Islet β-Cells MECHANISM OF β-CELL COMPENSATION AND FAILURE. J. Biol. Chem. 288, 25440-25449.

Gutierrez-Mazariegos, J., Theodosiou, M., Campo-Paysaa, F., Schubert, M., 2011. Vitamin A: A multifunctional tool for development, Semin. Cell Dev. Biol., Elsevier, pp. 603-610.

Hai, T., Hartman, M.G., 2001. The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. Gene 273, 1-11.

Hakuno, F., Yamauchi, Y., Kaneko, G., Yoneyama, Y., Nakae, J., Chida, K., Kadowaki, T., Yamanouchi, K., Nishihara, M., Takahashi, S., 2011. Constitutive expression of insulin receptor substrate (IRS)-1 inhibits myogenic differentiation through nuclear exclusion of Foxo1 in L6 myoblasts. PloS one 6, e25655.

Hales, C.N., Barker, D.J., 1992. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia 35, 595-601.

Halliwell, B., 1994. Free radicals and antioxidants: a personal view. Nutr. Rev. 52, 253-265.

Hamam, D., Ali, D., Vishnubalaji, R., Hamam, R., Al-Nbaheen, M., Chen, L., Kassem, M., Aldahmash, A., Alajez, N., 2014. microRNA-320/RUNX2 axis regulates adipocytic differentiation of human mesenchymal (skeletal) stem cells. Cell death & disease 5, e1499.

Hamilton, D.N., Ellis, M., Miller, K.D., McKeith, F.K., Parrett, D.F., 2000. The effect of the Halothane and Rendement Napole genes on carcass and meat quality characteristics of pigs. J. Anim. Sci. 78, 2862-2867.

Handschin, C., Spiegelman, B.M., 2006. Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism. Endocr. Rev. 27, 728-735.

Hannenhalli, S., Kaestner, K.H., 2009. The evolution of Fox genes and their role in development and disease. Nature Reviews Genetics 10, 233-240.

Harmon, B., Miller, E., Hoefer, J., Ullrey, D., Luecke, R., 1963. Relationship of Specific Nutrient Deficiencies to Antibody Production in Swine I. Vitamin A. The Journal of nutrition 79, 263-268.

Harper, G.S., Pethick, D., 2001. The physiology of marbling: what is it, and why does it develop.

Harris, R.B., 2014. Direct and indirect effects of leptin on adipocyte metabolism. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1842, 414-423.

Harrison, E.H., 2005. Mechanisms of digestion and absorption of dietary vitamin a*. Annu. Rev. Nutr. 25, 87-103.

Hartog, J.M., Verdouw, P.D., Klompe, M., Lamers, J., 1987. Dietary mackerel oil in pigs: effect on plasma lipids, cardiac sarcolemmal phospholipids and cardiovascular parameters. The Journal of nutrition 117, 1371-1378.

Hauser, N., Mourot, J., De Clercq, L., Genart, C., Remacle, C., 1997. The cellularity of developing adipose tissues in Pietrain and Meishan pigs. Reprod. Nutr. Dev. 37, 617-625.

Hausman, G., Thomas, G., 1986. Structural and histochemical aspects of perirenal adipose tissue in fetal pigs: Relationships between stromal- vascular characteristics and fat cell concentration and enzyme activity. J. Morphol. 190, 271-283.

Hausman, G.J., Basu, U., Du, M., Fernyhough-Culver, M., Dodson, M.V., 2014. Intermuscular and intramuscular adipose tissues: Bad vs. good adipose tissues. Adipocyte 3, 0--1.

Hausman, G.J., Campion, D.R., Martin, R.J., 1980. Search for the adipocyte precursor cell and factors that promote its differentiation. J. Lipid Res. 21, 657-670.

Hausman, G.J., Dodson, M.V., Ajuwon, K., Azain, M., Barnes, K.M., Guan, L.L., Jiang, Z., Poulos, S.P., Sainz, R.D., Smith, S., Spurlock, M., Novakofski, J., Fernyhough, M.E., Bergen, W.G., 2009. Board-invited review: the biology and regulation of preadipocytes and adipocytes in meat animals. J. Anim. Sci. 87, 1218-1246.

He, Q., Ren, P., Kong, X., Wu, Y., Wu, G., Li, P., Hao, F., Tang, H., Blachier, F., Yin, Y., 2012. Comparison of serum metabolite compositions between obese and lean growing pigs using an NMR-based metabonomic approach. The Journal of nutritional biochemistry 23, 133-139.

Herault, F., Vincent, A., Dameron, O., Le Roy, P., Cherel, P., Damon, M., 2014. The longissimus and semimembranosus muscles display marked differences in their gene expression profiles in pig.

Hernández, J., 2002. Nutrición vitamínica óptima en ganado porcino, In: Barroeta, A., Calsamiglia, S., Cepero, R., López-Bote, C., Hernández, J. (Eds.), Óptima nutrición vitamínica de los animales para la producción de alimentos de calidad., España. Editorial Pulso.

Herrera, E., 2002. Implications of dietary fatty acids during pregnancy on placental, fetal and postnatal development—a review. Placenta 23, S9-S19.

Herrera, E., Amusquivar, E., Lopez-Soldado, I., Ortega, H., 2006. Maternal lipid metabolism and placental lipid transfer. Hormone Research in Paediatrics 65, 59-64.

Hinson, R., Wiegand, B., Ritter, M., Allee, G., Carr, S., 2011. Impact of dietary energy level and ractopamine on growth performance, carcass characteristics, and meat quality of finishing pigs. J. Anim. Sci. 89, 3572-3579.

Hirata, M., Suzuki, M., Ishii, R., Satow, R., Uchida, T., Kitazumi, T., Sasaki, T., Kitamura, T., Yamaguchi, H., Nakamura, Y., 2011. Genetic defect in phospholipase Cδ1 protects mice from obesity by regulating thermogenesis and adipogenesis. Diabetes 60, 1926-1937.

Hoppe, P., Schöner, F., Frigg, M., 1991. Effects of dietary retinol on hepatic retinol storage and on plasma and tissue alpha-tocopherol in pigs. International journal for vitamin and nutrition research. Internationale Zeitschrift fur Vitamin-und Ernahrungsforschung. Journal international de vitaminologie et de nutrition 62, 121-129.

Horton, J.D., Goldstein, J.L., Brown, M.S., 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J. Clin. Invest. 109, 1125-1132.

Hsiao, S., Huang, K., Chang, H., Chen, S., 2009. P/CAF rescues the Bhlhe40-mediated repression of MyoD transactivation. Biochem. J 422, 343-352.

Hubbard, R., Wald, G., 1952. Cis-trans isomers of vitamin A and retinene in the rhodopsin system. The Journal of general physiology 36, 269-315.

Hudak, C.S., Sul, H.S., 2013. Pref-1, a gatekeeper of adipogenesis. Frontiers in endocrinology 4.

Hudson, N.J., Dalrymple, B.P., Reverter, A., 2012. Beyond differential expression: the quest for causal mutations and effector molecules. BMC Genomics 13, 356.

Huff, P.W., Lozeman, F.J., Weselake, R.J., Wegner, J., 2005. Immunohistochemical localization of preadipocyte factor- 1: potential marker of preadipocytes in bovine muscle tissue. Journal of Muscle Foods 16, 155-176.

Hulbert, A., Pamplona, R., Buffenstein, R., Buttemer, W., 2007. Life and death: metabolic rate, membrane composition, and life span of animals. Physiol. Rev. 87, 1175-1213.

Hulver, M.W., Berggren, J.R., Carper, M.J., Miyazaki, M., Ntambi, J.M., Hoffman, E.P., Thyfault, J.P., Stevens, R., Dohm, G.L., Houmard, J.A., 2005. Elevated stearoyl-CoA desaturase-1 expression in skeletal muscle contributes to abnormal fatty acid partitioning in obese humans. Cell metabolism 2, 251-261.

Hutley, L., Shurety, W., Newell, F., McGeary, R., Pelton, N., Grant, J., Herington, A., Cameron, D., Whitehead, J., Prins, J., 2004. Fibroblast Growth Factor 1 A Key Regulator of Human Adipogenesis. Diabetes 53, 3097-3106.

Hyun, Y., Ellis, M., McKeith, F., Baker, D., 2003. Effect of dietary leucine level on growth performance, and carcass and meat quality in finishing pigs. Canadian journal of animal science 83, 315-318.

Hyun, Y., Kim, J., Ellis, M., Peterson, B., Baker, D., McKeith, F., 2007. Effect of dietary leucine and lysine levels on intramuscular fat content in finishing pigs. Canadian journal of animal science 87, 303-306.

Ivana, L., Ohkawa, Y., Berkes, C.A., Bergstrom, D.A., Dacwag, C.S., Tapscott, S.J., Imbalzano, A.N., 2005. MyoD targets chromatin remodeling complexes to the myogenin locus prior to forming a stable DNA-bound complex. Mol. Cell. Biol. 25, 3997-4009.

Iwasaki, S., Miyake, M., Hayashi, S., Watanabe, H., Nagasawa, Y., Terada, S., Watanabe, K., Ohwada, S., Kitazawa, H., Rose, M.T., 2013. Effect of Myostatin on Chemokine Expression in Regenerating Skeletal Muscle Cells. Cells Tissues Organs 198, 66-74.

Izpisúa-Belmonte, J.-C., Duboule, D., 1992. Homeobox genes and pattern formation in the vertebrate limb. Dev. Biol. 152, 26-36.

Jacobs, K., Rohrer, G., Van Poucke, M., Piumi, F., Yerle, M., Barthenschlager, H., Mattheeuws, M., Van Zeveren, A., Peelman, L., 2006. Porcine PPARGC1A (peroxisome proliferative activated receptor gamma coactivator 1A): coding sequence, genomic organization, polymorphisms and mapping. Cytogenet. Genome Res. 112, 106-113.

Jaitovich, A., Angulo, M., Lecuona, E., Dada, L.A., Welch, L.C., Cheng, Y., Gusarova, G., Ceco, E., Liu, C., Shigemura, M., 2015. High CO2 Levels Cause Skeletal Muscle Atrophy via AMP-activated Kinase (AMPK), FoxO3a Protein, and Muscle-specific Ring Finger Protein 1 (MuRF1). J. Biol. Chem. 290, 9183-9194.

Jang, S.-M., Kim, J.-W., Kim, D., Kim, C.-H., An, J.-H., Choi, K.-H., Rhee, S., 2013. Sox4-mediated caldesmon expression facilitates differentiation of skeletal myoblasts. J. Cell Sci. 126, 5178-5188.

Jesse, T.L., LaChance, R., Iademarco, M.F., Dean, D.C., 1998. Interferon regulatory factor-2 is a transcriptional activator in muscle where it regulates expression of vascular cell adhesion molecule-1. The Journal of cell biology 140, 1265-1276.

Jeyakumar, S.M., Vajreswari, A., Giridharan, N.V., 2008. Vitamin A regulates obesity in WNIN/Ob obese rat; independent of stearoyl-CoA desaturase-1. Biochem. Biophys. Res. Commun. 370, 243-247.

Johnson, A.D., Zhang, Y., Papp, A.C., Pinsonneault, J.K., Lim, J.-E., Saffen, D., Dai, Z., Wang, D., Sadee, W., 2008. Polymorphisms affecting gene transcription and mRNA processing in pharmacogenetic candidate genes: detection through allelic expression imbalance in human target tissues. Pharmacogenetics and genomics 18, 781.

Johnson, R.N., Metcalf, P.A., Baker, J.R., 1983. Fructosamine: a new approach to the estimation of serum glycosylprotein. An index of diabetic control. Clinica Chimica Acta 127, 87-95.

Joyner, J., Hutley, L., Cameron, D., 2000. Glucocorticoid receptors in human preadipocytes: regional and gender differences. J. Endocrinol. 166, 145-152.

Kabir, S.M., Lee, E.-S., Son, D.-S., 2014. Chemokine network during adipogenesis in 3T3-L1 cells: Differential response between growth and proinflammatory factor in preadipocytes vs. adipocytes. Adipocyte 3, 97-106.

Kadmiel, M., Cidlowski, J.A., 2013. Glucocorticoid receptor signaling in health and disease. Trends Pharmacol. Sci. 34, 518-530.

Kamburov, A., Pentchev, K., Galicka, H., Wierling, C., Lehrach, H., Herwig, R., 2011. ConsensusPathDB: toward a more complete picture of cell biology. Nucleic Acids Res. 39, D712-D717.

Kamei, Y., Kawada, T., Kazuki, R., Sugimoto, E., 1993. Retinoic acid receptor gamma 2 gene expression is up-regulated by retinoic acid in 3T3-L1 preadipocytes. Biochem. J 293, 807-812.

Karastergiou, K., Fried, S.K., Xie, H., Lee, M.-J., Divoux, A., Rosencrantz, M.A., Chang, R.J., Smith, S.R., 2013. Distinct Developmental Signatures of Human Abdominal and Gluteal Subcutaneous Adipose Tissue Depots. J Clin Endocr Metab 98, 362-371.

Karlsson, A., Enfält, A.-C., Essén-Gustavsson, B., Lundström, K., Rydhmer, L., Stern, S., 1993. Muscle histochemical and biochemical properties in relation to meat quality during selection for increased lean tissue growth rate in pigs. J. Anim. Sci. 71, 930-938.

Katsumata, M., 2011. Promotion of intramuscular fat accumulation in porcine muscle by nutritional regulation. Animal science journal 82, 17-25.

Kawada, T., Aoki, N., Kamei, Y., Maeshige, K., Nishiu, S., Sugimoto, E., 1990. Comparative investigation of vitamins and their analogues on terminal differentiation, from preadipocytes to adipocytes, of 3T3-L1 cells. Comparative Biochemistry and Physiology Part A: Physiology 96, 323-326.

Kawada, T., Kamei, Y., Sugimoto, E., 1996. The possibility of active form of vitamins A and D as suppressors on adipocyte development via ligand-dependent transcriptional regulators. International journal of obesity and related metabolic disorders: journal of the International Association for the Study of Obesity 20, S52-57.

Kayan, A., Cinar, M., Uddin, M., Phatsara, C., Wimmers, K., Ponsuksili, S., Tesfaye, D., Looft, C., Juengst, H., Tholen, E., 2011. Polymorphism and expression of the porcine Tenascin C gene associated with meat and carcass quality. Meat science 89, 76-83.

Kerr, B., Easter, R., 1995. Effect of feeding reduced protein, amino acid-supplemented diets on nitrogen and energy balance in grower pigs. J. Anim. Sci. 73, 3000-3008.

Kiefer, F.W., Orasanu, G., Nallamshetty, S., Brown, J.D., Wang, H., Luger, P., Qi, N.R., Burant, C.F., Duester, G., Plutzky, J., 2012. Retinaldehyde dehydrogenase 1 coordinates hepatic gluconeogenesis and lipid metabolism. Endocrinology 153, 3089-3099.

Kim, H., Hausman, D., Compton, M., Dean, R., Martin, R., Hausman, G., Hartzell, D., Baile, C., 2000. Induction of Apoptosis by All-trans-Retinoic Acid and C2-Ceramide Treatment in Rat Stromal–Vascular Cultures. Biochem. Biophys. Res. Commun. 270, 76-80.

Kim, J., Seong, P., Cho, S., Park, B., Hah, K., Yu, L., Lim, D., Hwang, I., Kim, D., Lee, J., 2008. Characterization of nutritional value for twenty-one pork muscles. Asian australasian journal of animal sciences. 21, 138.

Kim, J.B., Spiegelman, B.M., 1996. ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. Genes Dev. 10, 1096-1107.

Kim, N.-K., Park, H.-R., Lee, H.-C., Yoon, D., Son, E.-S., Kim, Y.-S., Kim, S.-R., Kim, O.-H., Lee, C.-S., 2010. Comparative studies of skeletal muscle proteome and transcriptome profilings between pig breeds. Mamm. Genome 21, 307-319.

Kim, S.W., Il Choi, Y., Choi, J.S., Kim, J.J., Choi, B.H., Kim, T.H., Kim, K.S., 2011. Porcine Fatty Acid Synthase Gene Polymorphisms Are Associated with Meat Quality and Fatty Acid Composition. Korean J Food Sci An 31, 356-365.

Klem, T.B., Bleken, E., Morberg, H., Thoresen, S.I., Framstad, T., 2010. Hematologic and biochemical reference intervals for Norwegian crossbreed grower pigs. Veterinary Clinical Pathology 39, 221-226.

Koc, M., Mayerová, V., Kračmerová, J., Mairal, A., Mališová, L., Štich, V., Langin, D., Rossmeislová, L., 2015. Stress of endoplasmic reticulum modulates differentiation and lipogenesis of human adipocytes. Biochem. Biophys. Res. Commun. 460, 684-690.

Könner, A.C., Klöckener, T., Brüning, J.C., 2009. Control of energy homeostasis by insulin and leptin: targeting the arcuate nucleus and beyond. Physiol. Behav. 97, 632-638.

Korner, J., Savontaus, E., Chua, S., Leibel, R., Wardlaw, S., 2001. Leptin regulation of Agrp and Npy mRNA in the rat hypothalamus. J. Neuroendocrinol. 13, 959-966.

Kouba, M., Mourot, J., 2011. A review of nutritional effects on fat composition of animal products with special emphasis on n-3 polyunsaturated fatty acids. Biochimie 93, 13-17.

Kristensen, P., Judge, M.E., Thim, L., Ribel, U., Christjansen, K.N., Wulff, B.S., Clausen, J.T., Jensen, P.B., Madsen, O.D., Vrang, N., 1998. Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393, 72-76.

Kruk, Z., Bottema, C.D.K., Davis, J., Siebert, B.D., Harper, G.S., Di, J., Pitchford, W.S., 2008. Effects of vitamin A on growth performance and carcass quality in steers. Livest Sci 119, 12-21.

Kurebayashi, S., Hirose, T., Miyashita, Y., Kasayama, S., Kishimoto, T., 1997. Thiazolidinediones downregulate stearoyl-CoA desaturase 1 gene expression in 3T3-L1 adipocytes. Diabetes 46, 2115-2118.

Kuri-Harcuch, W., 1982. Differentiation of 3T3-F442A cells into adipocytes is inhibited by retinoic acid. Differentiation 23, 164-169.

Kuwata, T., Wang, I.M., Tamura, T., Ponnamperuma, R.M., Levine, R., Holmes, K.L., Morse, H.C., De Luca, L.M., Ozato, K., 2000. Vitamin A deficiency in mice causes a systemic expansion of myeloid cells. Blood 95, 3349-3356.

Larzul, C., Lefaucheur, L., Ecolan, P., Gogue, J., 1997. Phenotypic and genetic parameters for longissimus muscle fiber characteristics in relation to growth, carcass, and meat quality traits in large white pigs. J. Anim. Sci. 75, 3126.

Latorre, M., Lázaro, R., Gracia, M., Nieto, M., Mateos, G., 2003a. Effect of sex and terminal sire genotype on performance, carcass characteristics, and meat quality of pigs slaughtered at 117 kg body weight. Meat science 65, 1369-1377.

Latorre, M., Medel, P., Fuentetaja, A., Lázaro, R., Mateos, G., 2003b. Effect of gender, terminal sire line and age at slaughter on performance, carcass characteristics and meat quality of heavy pigs. Animal science-glasgow then penicuik. 77, 33-46.

Latorre, M., Lázaro, R., Valencia, D., Medel, P., Mateos, G., 2004. The effects of gender and slaughter weight on the growth performance, carcass traits, and meat quality characteristics of heavy pigs. J. Anim. Sci. 82, 526-533.

Lebret, B., Juin, H., Noblet, J., Bonneau, M., 2001. The effects of two methods of increasing age at slaughter on carcass and muscle traits and meat sensory quality in pigs. Animal Science 72, 87-94.

Lee, K., Buhr, J., Hausman, G.J., Wright, T., Dean, R., 1996. Expression of c-Fos in subcutaneous adipose tissue of the fetal pig. Mol. Cell. Biochem. 155, 31-35.

Lee, K., Villena, J.A., Moon, Y.S., Kim, K.-H., Lee, S., Kang, C., Sul, H.S., 2003. Inhibition of adipogenesis and development of glucose intolerance by soluble preadipocyte factor–1 (Pref-1). J. Clin. Invest. 111, 453.

Lee, S.-H., Lee, S., Yang, H., Song, S., Kim, K., Saunders, T.L., Yoon, J.K., Koh, G.Y., Kim, I., 2014. Notch pathway targets proangiogenic regulator Sox17 to restrict angiogenesis. Circul. Res. 115, 215-226.

Lefaucheur, L., Lebret, B., Ecolan, P., Louveau, I., Damon, M., Prunier, A., Billon, Y., Sellier, P., Gilbert, H., 2011. Muscle characteristics and meat quality traits are affected by divergent selection on residual feed intake in pigs. J. Anim. Sci. 89, 996-1010.

Lei, H., Leong, D., Smith, L.R., Barton, E.R., 2013. Matrix metalloproteinase 13 is a new contributor to skeletal muscle regeneration and critical for myoblast migration. American Journal of Physiology-Cell Physiology 305, C529-C538.

Leow, M., Addy, C., Mantzoros, C., 2003. Human immunodeficiency virus/highly active antiretroviral therapy-associated metabolic syndrome: clinical presentation, pathophysiology, and therapeutic strategies. J. Clin. Endocrinol. Metab. 88, 1961-1976.

Leseigneur-Meynier, A., Gandemer, G., 1991. Lipid composition of pork muscle in relation to the metabolic type of the fibres. Meat Science 29, 229-241.

Li, W., Zhao, S., Huang, Y., Yang, M., Pan, H., Zhang, X., Ge, C., Gao, S., 2012. Expression of lipogenic genes during porcine intramuscular preadipocyte differentiation. Res. Vet. Sci. 93, 1190-1194.

Lin, F.-T., Lane, M.D., 1992. Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. Genes Dev. 6, 533-544.

Lin, H.-Q., Choi, R., Chan, K.-L., Ip, D., Tsim, K.W.-k., Wan, D.C.-c., 2010. Differential gene expression profiling on the muscle of acetylcholinesterase knockout mice: A preliminary analysis. Chem.-Biol. Interact. 187, 120-123.

Lindemann, M., Brendemuhl, J., Chiba, L., Darroch, C., Dove, C., Estienne, M., Harper, A., 2008. A regional evaluation of injections of high levels of vitamin A on reproductive performance of sows. J. Anim. Sci. 86, 333-338.

Linhart, H.G., Ishimura-Oka, K., DeMayo, F., Kibe, T., Repka, D., Poindexter, B., Bick, R.J., Darlington, G.J., 2001. CEBPA is required for differentiation of white, but not brown, adipose tissue. Proceedings of the National Academy of Sciences 98, 12532-12537.

Liu, B.H., Wang, Y.C., Wu, S.C., Mersmann, H.J., Cheng, W.T., Ding, S.T., 2008. Insulin regulates the expression of adiponectin and adiponectin receptors in porcine adipocytes. Domest. Anim. Endocrinol. 34, 352-359.

Liu, J., Sridhar, J., Foroozesh, M., 2013. Cytochrome P450 family 1 inhibitors and structure-activity relationships. Molecules 18, 14470-14495.

Liu, Z., Yang, F., Kong, L., Zhou, X., Gu, Y., Wang, X., 2007. Effects of dietary energy level on the content of intramuscular fat and mRNA expression for fatty acid synthase and hormone-sensitive lipase in growing-finishing pigs.

Lo Fiego, D.P., Macchioni, P., Minelli, G., Santoro, P., 2010. Lipid composition of covering and intramuscular fat in pigs at different slaughter age. Ital J Anim Sci 9, 200-205.

Lodish, H.F., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D., Darnell, J., 2000. Molecular cell biology. Citeseer.

López-Bote, C., 1998. Sustained utilization of the Iberian pig breed. Meat science 49, S17-S27.

López-Bote, C., Toldrá, F., Daza, A., Ferrer, J., Menoyo, D., Silió, L., Rodríguez, M., 2008. Effect of exercise on skeletal muscle proteolytic enzyme activity and meat quality characteristics in Iberian pigs. Meat science 79, 71-76.

Lopez-Bote, C.J., Rey, A.I., Isabel, B., Sanz, R., 1997. Effect of feeding diets high in monounsaturated fatty acids and alpha-tocopheryl acetate to rabbits on resulting carcass fatty acid profile and lipid oxidation. Anim. Sci. 64, 177-186.

Lujan, R., Shigemoto, R., Lopez-Bendito, G., 2005. Glutamate and GABA receptor signalling in the developing brain. Neuroscience 130, 567-580.

Luo, X., Yu, C., Fu, C., Shi, W., Wang, X., Zeng, C., Wang, H., 2015. Identification of the differentially expressed genes associated with familial combined hyperlipidemia using bioinformatics analysis. Molecular medicine reports 11, 4032-4038.

Ma, X., Lee, P., Chisholm, D.J., James, D.E., 2015. Control of adipocyte differentiation in different fat depots; implications for pathophysiology or therapy. Frontiers in endocrinology 6.

MacDougald, O.A., Lane, M.D., 1995. Transcriptional regulation of gene expression during adipocyte differentiation. Annu. Rev. Biochem. 64, 345-373.

Mach, N., Gao, Y., Lemonnier, G., Lecardonnel, J., Oswald, I.P., Estellé, J., Rogel-Gaillard, C., 2013. The peripheral blood transcriptome reflects variations in immunity traits in swine: towards the identification of biomarkers. BMC Genomics 14, 894.

Madsen, A., Jakobsen, K., Mortensen, H.P., 1992. Influence of dietary fat on carcass fat quality in pigs. A review. Acta Agriculturae Scandinavica A-Animal Sciences 42, 220-225.

Maekawa, T., Jin, W., Ishii, S., 2010. The role of ATF-2 family transcription factors in adipocyte differentiation: antiobesity effects of p38 inhibitors. Mol. Cell. Biol. 30, 613-625.

Mamula, P.W., McDonald, A.R., Brunetti, A., Okabayashi, Y., Wong, K.Y., Maddux, B.A., Logsdon, C., Goldfine, I.D., 1990. Regulating insulin-receptor-gene expression by differentiation and hormones. Diabetes Care 13, 288-301.

Mantione, K.J., Kream, R.M., Kuzelova, H., Ptacek, R., Raboch, J., Samuel, J.M., Stefano, G.B., 2014. Comparing bioinformatic gene expression profiling methods: Microarray and RNA-Seq. Medical science monitor basic research 20, 138.

Margareto, J., Gómez-Ambrosi, J., Marti, A., Martínez, J.A., 2001. Time-dependent effects of a high-energy-yielding diet on the regulation of specific white adipose tissue genes. Biochem. Biophys. Res. Commun. 283, 6-11.

Marion, V., Sankaranarayanan, S., de Theije, C., van Dijk, P., Hakvoort, T.B., Lamers, W.H., Köhler, E.S., 2013. Hepatic adaptation compensates inactivation of intestinal arginine biosynthesis in suckling mice.

Marioni, J., Mason, C., Mane, S., Stephens, M., Gilad, Y., 2008. RNA-seq: An assessment of technical reproducibility and comparison with gene expression arrays. Genome Res. 18, 1509-1517.

Marti, S., Realini, C., Bach, A., Pérez-Juan, M., Devant, M., 2011. Effect of vitamin A restriction on performance and meat quality in finishing Holstein bulls and steers. Meat science 89, 412-418.

Martin, J.A., Wang, Z., 2011. Next-generation transcriptome assembly. Nature Reviews Genetics 12, 671-682.

Mata, P., Alonso, R., Ruíz-Garcia, A., Díaz-Díaz, J.L., González, N., Gijón-Conde, T., Martínez-Faedo, C., Morón, I., Arranz, E., Aguado, R., 2014. Hiperlipidemia familiar combinada: documento de consenso. Atención Primaria 46, 440-446.

Mayoral, A., Dorado, M., Guillén, M., Robina, A., Vivo, J., Vázquez, C., Ruiz, J., 1999. Development of meat and carcass quality characteristics in Iberian pigs reared outdoors. Meat Science 52, 315-324.

McPherron, A.C., Lawler, A.M., Lee, S.-J., 1997. Regulation of skeletal muscle mass in mice by a new TGF-p superfamily member.

Merbitz-Zahradnik, T., Wolf, E., 2015. How is the inner Circadian Clock controlled by interactive clock proteins? FEBS Lett.

Meruane, M., Rojas, M., 2010. Células troncales derivadas del tejido adiposo. International Journal of Morphology 28, 879-889.

Miller, C.W., Waters, K.M., Ntambi, J.M., 1997. Regulation of hepatic stearoyl-CoA desaturase gene 1 by vitamin A. Biochem. Biophys. Res. Commun. 231, 206-210.

Miller, M., Shackelford, S., Hayden, K., Reagan, J., 1990. Determination of the alteration in fatty acid profiles, sensory characteristics and carcass traits of swine fed elevated levels of monounsaturated fats in the diet. J. Anim. Sci. 68, 1624-1631.

Miller, M.R., Zhang, W., Sibbel, S.P., Langefeld, C.D., Bowden, D.W., Haffner, S.M., Bergman, R.N., Norris, J.M., Fingerlin, T.E., 2010. Variant in the 3′ Region of the IκBα Gene Associated With Insulin Resistance in Hispanic Americans: The IRAS Family Study. Obesity 18, 555-562.

Miron, M., Woody, O.Z., Marcil, A., Murie, C., Sladek, R., Nadon, R., 2006. A methodology for global validation of microarray experiments. BMC Bioinformatics 7, 333.

Mizugishi, K., Hatayama, M., Tohmonda, T., Ogawa, M., Inoue, T., Mikoshiba, K., Aruga, J., 2004. Myogenic repressor I-mfa interferes with the function of Zic family proteins. Biochem. Biophys. Res. Commun. 320, 233-240.

Moinard, C., Le Plenier, S., Noirez, P., Morio, B., Bonnefont-Rousselot, D., Kharchi, C., Ferry, A., Neveux, N., Cynober, L., Raynaud-Simon, A., 2015. Citrulline Supplementation Induces Changes in Body Composition and Limits Age-Related Metabolic Changes in Healthy Male Rats. The Journal of Nutrition, jn200626.

Molotkov, A., Duester, G., 2003. Genetic evidence that retinaldehyde dehydrogenase Raldh1 (Aldh1a1) functions downstream of alcohol dehydrogenase Adh1 in metabolism of retinol to retinoic acid. J. Biol. Chem. 278, 36085-36090.

Monahan, F.J., Gray, J.I., Asghar, A., Haug, A., Strasburg, G.M., Buckley, D.J., Morrissey, P.A., 1994. Influence of diet on lipid oxidation and membrane structure in porcine muscle microsomes. J. Agric. Food Chem. 42, 59-63.

Moon, Y.S., Smas, C.M., Lee, K., Villena, J.A., Kim, K.-H., Yun, E.J., Sul, H.S., 2002. Mice lacking paternally expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity. Mol. Cell. Biol. 22, 5585-5592.

Moreno-Aliaga, M.J., Martinez, J.A., 2002. El tejido adiposo: órgano de almacenamiento y órgano secretor.

Moreno-Navarrete, J.M., Fernández-Real, J.M., 2011. Adipocyte Differentiation, In: Symonds, M.E. (Ed.), Adipose tissue biology, Springer Science & Business Media, pp. 17-38.

Mori, M., Nakagami, H., Rodriguez-Araujo, G., Nimura, K., Kaneda, Y., 2012. Essential Role for miR-196a in Brown Adipogenesis of White Fat Progenitor Cells. PLoS Biol. 10.

Morris, J., 2001. Genes, genetics, and epigenetics: a correspondence. Science 293, 1103-1105.

Morrison, R.F., Farmer, S.R., 2000. Hormonal signaling and transcriptional control of adipocyte differentiation. The Journal of nutrition 130, 3116S-3121S.

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., Wold, B., 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5, 621-628.

Mourot, J., 2001. Mise en place des tissus adipeux sous-cutanés et intramusculaires et facteurs de variation quantitatifs et qualitatifs chez le porc. Prod Anim 14, 355-363.

Mozaffarian, D., Wu, J.H., 2011. Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events. J. Am. Coll. Cardiol. 58, 2047-2067.

Munoz, J., Lok, K.H., Gower, B.A., Fernandez, J.R., Hunter, G.R., Lara-Castro, C., De Luca, M., Garvey, W.T., 2006. Polymorphism in the transcription factor 7-like 2 (TCF7L2) gene is associated with reduced insulin secretion in nondiabetic women. Diabetes 55, 3630-3634.

Muñoz, G., Ovilo, C., Silió, L., Tomás, A., Noguera, J., Rodríguez, M., 2009. Single-and joint-population analyses of two experimental pig crosses to confirm quantitative trait loci on chromosome 6 and leptin receptor effects on fatness and growth traits. J. Anim. Sci. 87, 459-468.

Murgiano, L., D'Alessandro, A., Egidi, M.G., Crisa, A., Prosperini, G., Timperio, A.M., Valentini, A., Zolla, L., 2010. Proteomics and transcriptomics investigation on longissimus muscles in Large White and Casertana pig breeds. Journal of proteome research 9, 6450-6466.

Muriel, E., Ruiz, J., Ventanas, J., Petron, M.J., Antequera, T., 2004a. Meat quality characteristics in different lines of Iberian pigs. Meat Science 67, 299-307.

Murphy, K.T., Ham, D.J., Church, J.E., Naim, T., Trieu, J., Williams, D.A., Lynch, G.S., 2012. Parvalbumin gene transfer impairs skeletal muscle contractility in old mice. Hum. Gene Ther. 23, 824-836.

Myers, M.G., Cowley, M.A., Münzberg, H., 2008. Mechanisms of leptin action and leptin resistance. Annu. Rev. Physiol. 70, 537-556.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esuml, E., Li, S., Nonaka, I., Nabeshima, Y.-i., 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect.

Nakamura, M., Nara, T., 2002. Gene regulation of mammalian dasaturases. Biochem. Soc. Trans. 30, 1076-1079.

Neal, J.W., Clipstone, N.A., 2003. A constitutively active NFATc1 mutant induces a transformed phenotype in 3T3-L1 fibroblasts. J. Biol. Chem. 278, 17246-17254.

Nechtelberger, D., Pires, V., Solkner, J., Stur, I., Brem, G., Mueller, M., Mueller, S., 2001. Intramuscular fat content and genetic variants at fatty acid-binding protein loci in Austrian pigs. Journal of animal science-menasha then albany then champaign illinois- 79, 2798-2804.

Ng, P.C., Henikoff, S., 2003. SIFT: Predicting amino acid changes that affect protein function. Nucleic Acids Res. 31, 3812-3814.

Nilzén, V., Babol, J., Dutta, P., Lundeheim, N., Enfält, A., Lundström, K., 2001. Free range rearing of pigs with access to pasture grazing—effect on fatty acid composition and lipid oxidation products. Meat Science 58, 267-275.

Nishide, R., Ando, M., Funabashi, H., Yoda, Y., Nakano, M., Shima, M., 2015. Association of serum hs-CRP and lipids with obesity in school children in a 12-month follow-up study in Japan. Environ. Health Prevent. Med. 20, 116-122.

Nishizawa, S., Koya, T., Ohno, Y., Goto, A., Ikuita, A., Suzuki, M., Ohira, T., Egawa, T., Nakai, A., Sugiura, T., 2013. Regeneration of injured skeletal muscle in heat shock transcription factor 1- null mice. Physiological reports 1, e00071.

Noy, N., 2013. The one-two punch: Retinoic acid suppresses obesity both by promoting energy expenditure and by inhibiting adipogenesis. Adipocyte 2, 184-187.

NRC, 1998. Nutrient requirements of swine (10th ed). Washington DC: National Academic Press.

NRC. 2012. Nutrient Requirements of Swine: Eleventh Revised Edition. The National Academies Press.

Ntambi, J.M., Kim, Y.C., 2000. Adipocyte differentiation and gene expression. J. Nutr. 130, 3122s-3126s.

O'Hea, E.K., Leveille, G.A., 1969. Significance of adipose tissue and liver as sites of fatty acid synthesis in the pig and the efficiency of utilization of various substrates for lipogenesis. The Journal of nutrition 99, 338-344.

Oishi, Y., Manabe, I., Tobe, K., Tsushima, K., Shindo, T., Fujiu, K., Nishimura, G., Maemura, K., Yamauchi, T., Kubota, N., 2005. Krüppel-like transcription factor KLF5 is a key regulator of adipocyte differentiation. Cell metabolism 1, 27-39.

Oka, A., Maruo, Y., Miki, T., Yamasaki, T., Saito, T., 1998. Influence of vitamin A on the quality of beef from the Tajima strain of Japanese black cattle. Meat Science 48, 159-167.

Oku, H., Tokuda, M., Okumura, T., Umino, T., 2006. Effects of insulin, triiodothyronine and fat soluble vitamins on adipocyte differentiation and LPL gene expression in the stromal-vascular cells of red sea bream,< i> Pagrus major</i>. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 144, 326-333.

Olivares, A., Daza, A., Rey, A.I., Lopez-Bote, C.J., 2009a. Dietary vitamin A concentration alters fatty acid composition in pigs. Meat Science 81, 295-299.

Olivares, A., Daza, A., Rey, A.I., Lopez-Bote, C.J., 2009b. Interactions between genotype, dietary fat saturation and vitamin A concentration on intramuscular fat content and fatty acid composition in pigs. Meat Science 82, 6-12.

Olivares, A., Rey, A.I., Daza, A., Lopez-Bote, C.J., 2009c. High dietary vitamin A interferes with tissue alpha-tocopherol concentrations in fattening pigs: a study that examines administration and withdrawal times. Animal 3, 1264-1270.

Olivares, A., Rey, A., Daza, A., López-Bote, C., 2011. Low levels of dietary vitamin A increase intramuscular fat content and polyunsaturated fatty acid proportion in liver from lean pigs. Livest Sci 137, 31-36.

Ollivier, L., 2009. European pig genetic diversity: a minireview. Animal 3, 915-924.

Olson, J.A., 1961. The absorption of beta-carotene and its conversion into vitamin A. The American journal of clinical nutrition 9, 1-12.

Oshlack, A., Robinson, M.D., Young, M.D., 2010. From RNA-seq reads to differential expression results. Genome biol 11, 220.

Osses, N., Brandan, E., 2002. ECM is required for skeletal muscle differentiation independently of muscle regulatory factor expression. American Journal of Physiology-Cell Physiology 282, C383-C394.

Ovilo, C., Benitez, R., Fernandez, A., Isabel, B., Nunez, Y., Fernandez, A.I., Rodriguez, C., Daza, A., Silio, L., Lopez-Bote, C., 2014a. Dietary energy source largely affects tissue fatty acid composition but has minor influence on gene transcription in Iberian pigs. J. Anim. Sci. 92, 939-954.

Ovilo, C., Benitez, R., Fernandez, A., Nunez, Y., Ayuso, M., Fernandez, A., Rodriguez, C., Isabel, B., Rey, A., Lopez-Bote, C., Silio, L., 2014b. Longissimus dorsi transcriptome analysis of purebred and crossbred Iberian pigs differing in muscle characteristics. BMC Genomics 15, 413.

Óvilo, C., Fernández, A., Fernández, A.I., Folch, J.M., Varona, L., Benítez, R., Nuñez, Y., Rodríguez, C., Silió, L., 2010. Hypothalamic expression of porcine leptin receptor (LEPR), neuropeptide Y (NPY), and cocaine-and amphetamine-regulated transcript (CART) genes is influenced by LEPR genotype. Mamm. Genome 21, 583-591.

Ovilo, C., Fernández, A., Noguera, J., Barragán, C., Letón, R., Rodríguez, C., Mercadé, A., Alves, E., Folch, J., Varona, L., 2005. Fine mapping of porcine chromosome 6 QTL and LEPR effects on body composition in multiple generations of an Iberian by Landrace intercross. Genet. Res. 85, 57-67.

Ovilo, C., González-Bulnes, A., Benítez, R., Ayuso, M., Barbero, A., Pérez-Solana, M.L., Barragán, C., Astiz, S., Fernández, A., López-Bote, C., 2014c. Prenatal programming in an obese swine model: sex-related effects of maternal energy restriction on morphology, metabolism and hypothalamic gene expression. British Journal of Nutrition 111, 735-746.

Pajukanta, P., Lilja, H.E., Sinsheimer, J.S., Cantor, R.M., Lusis, A.J., Gentile, M., Duan, X.J., Soro-Paavonen, A., Naukkarinen, J., Saarela, J., 2004. Familial combined hyperlipidemia is associated with upstream transcription factor 1 (USF1). Nat. Genet. 36, 371-376.

Palinski, W., 2009. Maternal–Fetal Cholesterol Transport in the Placenta Good, Bad, and Target for Modulation. Circul. Res. 104, 569-571.

Palstra, A.P., Rovira, M., Rizo, D., Torrella, J.R., Spaink, H.P., Planas, J.V., 2014. Swimming-induced exercise promotes hypertrophy and vascularization of fast skeletal muscle fibres and activation of myogenic and angiogenic transcriptional programs in adult zebrafish. BMC Genomics 15, 1136.

Pan, H., Gustafsson, M.K., Aruga, J., Tiedken, J.J., Chen, J.C., Emerson, C.P., 2011. A role for Zic1 and Zic2 in Myf5 regulation and somite myogenesis. Dev. Biol. 351, 120-127.

Pares, X., Farres, J., Kedishvili, N., Duester, G., 2008. Medium-and short-chain dehydrogenase/reductase gene and protein families. Cell. Mol. Life Sci. 65, 3936-3949.

Park, U.-H., Yoon, S.K., Park, T., Kim, E.-J., Um, S.-J., 2011. Additional sex comb-like (ASXL) proteins 1 and 2 play opposite roles in adipogenesis via reciprocal regulation of peroxisome proliferator-activated receptor G. J. Biol. Chem. 286, 1354-1363.

Paton, C.M., Ntambi, J.M., 2009. Biochemical and physiological function of stearoyl-CoA desaturase. Am J Physiol-Endoc M 297, E28-E37.

Peckett, A.J., Wright, D.C., Riddell, M.C., 2011. The effects of glucocorticoids on adipose tissue lipid metabolism. Metabolism 60, 1500-1510.

Pena, R., Noguera, J., Casellas, J., Díaz, I., Fernández, A., Folch, J., Ibáñez- Escriche, N., 2013. Transcriptional analysis of intramuscular fatty acid composition in the longissimus thoracis muscle of Iberian× Landrace back- crossed pigs. Anim. Genet. 44, 648-660.

Pérez-Enciso, M., Ferraz, A.L., Ojeda, A., López-Béjar, M., 2009. Impact of breed and sex on porcine endocrine transcriptome: a bayesian biometrical analysis. BMC Genomics 10, 89.

Pérez-Montarelo, D., Madsen, O., Alves, E., Rodríguez, M.C., Folch, J.M., Noguera, J.L., Groenen, M.A., Fernández, A.I., 2014. Identification of genes regulating growth and fatness traits in pig through hypothalamic transcriptome analysis. Physiol. Genomics 46, 195-206.

Pethick, D.W., Harper, G.S., Hocquette, J.-F., Wang, Y.H., 2006. Marbling biology - what do we know about getting fat into muscle?, Australian Beef - The leader conference! The impact of science on the beef industry, CRC for Beef Genetic Technologies, Armidale, Australia, pp. 103-110.

Petrón, M., Muriel, E., Timón, M., Martın, L., Antequera, T., 2004. Fatty acids and triacylglycerols profiles from different types of Iberian dry-cured hams. Meat Science 68, 71-77.

Picard, B., Lefaucheur, L., Berri, C., Duclos, M.J., 2002. Muscle fibre ontogenesis in farm animal species. Reprod. Nutr. Dev. 42, 415-431.

Pickworth, C.L., Loerch, S.C., Fluharty, F.L., 2012. Effects of timing and duration of dietary vitamin A reduction on carcass quality of finishing beef cattle. J. Anim. Sci. 90, 2677-2691.

Pomar, C., Bailleul, P.D., 2000. Determinación de las necesidades nutricionales de los cerdos de engorde: límites de los métodos actuales. Produccion Animal.

Ponsuksili, S., Murani, E., Brand, B., Schwerin, M., Wimmers, K., 2011. Integrating expression profiling and whole-genome association for dissection of fat traits in a porcine model. J. Lipid Res. 52, 668-678.

Posey, A.D., Swanson, K.E., Alvarez, M.G., Krishnan, S., Earley, J.U., Band, H., Pytel, P., McNally, E.M., Demonbreun, A.R., 2014. EHD1 mediates vesicle trafficking required for normal muscle growth and transverse tubule development. Dev. Biol. 387, 179-190.

Possemato, R., Marks, K.M., Shaul, Y.D., Pacold, M.E., Kim, D., Birsoy, K., Sethumadhavan, S., Woo, H.-K., Jang, H.G., Jha, A.K., 2011. Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. Nature 476, 346-350.

Poulos, S.P., Hausman, D.B., Hausman, G.J., 2010. The development and endocrine functions of adipose tissue. Mol. Cell. Endocrinol. 323, 20-34.

Puig-Oliveras, A., Ramayo-Caldas, Y., Corominas, J., Estellé, J., Pérez-Montarelo, D., Hudson, N.J., Casellas, J., Folch, J.M., Ballester, M., 2014. Differences in muscle transcriptome among pigs phenotypically extreme for fatty acid composition. Plos One 9, 11.

Pushpakom, S.P., Owen, A., Back, D.J., Pirmohamed, M., 2013. RXRG gene variants are associated with HIV lipodystrophy. Pharmacogenetics and genomics 23, 438-441.

Qian, X., Ba, Y., Zhuang, Q., Zhong, G., 2014. RNA-Seq technology and its application in fish transcriptomics. OMICS: J. Integrative Biol. 18, 98-110.

Quiniou, N., Richard, S., Mourot, J., Etienne, M., 2008. Effect of dietary fat or starch supply during gestation and/or lactation on the performance of sows, piglets' survival and on the performance of progeny after weaning. Anim. Feed Sci. Technol. 2, 1633-1644.

Raj, S., Skiba, G., Weremko, D., Fandrejewski, H., Migdal, W., Borowiec, F., Polawska, E., 2010. The relationship between the chemical composition of the carcass and the fatty acid composition of intramuscular fat and backfat of several pig breeds slaughtered at different weights. Meat Science 86, 324-330.

Ramayo-Caldas, Y., Mach, N., Esteve-Codina, A., Corominas, J., Castello, A., Ballester, M., Estelle, J., Ibanez-Escriche, N., Fernandez, A.I., Perez-Enciso, M., Folch, J.M., 2012. Liver transcriptome profile in pigs with extreme phenotypes of intramuscular fatty acid composition. BMC Genomics 13.

Ramsay, T., White, M., Wolverton, C., 1989. Insulin-like growth factor 1 induction of differentiation of porcine preadipocytes. J. Anim. Sci. 67, 2452-2459.

Reed, B.C., Lane, M.D., 1980. Insulin receptor synthesis and turnover in differentiating 3T3-L1 preadipocytes. Proceedings of the National Academy of Sciences 77, 285-289.

Ren, W., Guo, J., Jiang, F., Lu, J., Ding, Y., Li, A., Liang, X., Jia, W., 2014. CCAAT/enhancer-binding protein α is a crucial regulator of human fat mass and obesity associated gene transcription and expression. BioMed research international 2014.

Reverter, A., Hudson, N.J., Nagaraj, S.H., Pérez-Enciso, M., Dalrymple, B.P., 2010. Regulatory impact factors: unraveling the transcriptional regulation of complex traits from expression data. Bioinformatics 26, 896-904.

Rey, A., Daza, A., López-Carrasco, C., López-Bote, C., 2006a. Feeding Iberian pigs with acorns and grass in either free-range or confinement affects the carcass characteristics and fatty acids and tocopherols accumulation in Longissimus dorsi muscle and backfat. Meat science 73, 66-74.

Rey, A., Daza, A., López-Carrasco, C., López-Bote, C., 2006b. Quantitative study of the α-and G-tocopherols accumulation in muscle and backfat from Iberian pigs kept free-range as affected by time of free-range feeding or weight gain. Animal Science 82, 901-908.

Rey, A., Lopez-Bote, C., Kerry, J., Lynch, P., Buckley, D., Morrissey, P., 2004. Modification of lipid composition and oxidation in porcine muscle and muscle microsomes as affected by dietary supplementation of n-3 with either n-9 or n-6 fatty acids and α-tocopheryl acetate. Anim. Feed Sci. Technol. 113, 223-238.

Rey, A., Lopez-Bote, C., Sanz Arias, R., 1997. Effect of extensive feeding on α-tocopherol concentration and oxidative stability of muscle microsomes from Iberian pigs. Animal Science 65, 515-520.

Rey, A., López- Bote, C., 2001. Effect of dietary copper and vitamin E supplementation, and extensive feeding with acorn and grass on longissimus muscle composition and susceptibility to oxidation in Iberian pigs. J. Anim. Physiol. Anim. Nutr. 85, 281-292.

Ribot, J., Felipe, F., Bonet, M.L., Palou, A., 2001. Changes of adiposity in response to vitamin A status correlate with changes of PPARG2 expression. Obesity Res. 9, 500-509.

Ribot, J., Serra, F., Palou, A., 2002. Retinoic acid decreases the expression and phosphorylation state of the retinoblastoma protein in adipocyte terminal differentiation. Int. J. Obes. Relat. Metab. Disord 26.

Rivera-Ferre, M., Aguilera, J., Nieto, R., 2005. Muscle fractional protein synthesis is higher in Iberian than in Landrace growing pigs fed adequate or lysine-deficient diets. The Journal of nutrition 135, 469-478.

Robina, A., Viguera, J., Perez-Palacios, T., Mayoral, A.I., Vivo, J.M., Guillen, M.T., Ruiz, J., 2013. Carcass and meat quality traits of Iberian pigs as affected by sex and crossbreeding with different Duroc genetic lines. Span J Agric Res 11, 1057-1067.

Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139-140.

Rodriguez-Sanchez, J.A., Ripoll, G., Latorre, M.A., 2010. The influence of age at the beginning of Montanera period on meat characteristics and fat quality of outdoor Iberian pigs. Animal 4, 289-294.

Ropka-Molik, K., Zukowski, K., Eckert, R., Gurgul, A., Piorkowska, K., Oczkowicz, M., 2014. Comprehensive analysis of the whole transcriptomes from two different pig breeds using RNA-Seq method. Anim. Genet. 45, 674-684.

Rosc, D., Adamczyk, P., Boinska, J., Szafkowski, R., Ponikowska, I., Stankowska, K., Goralczyk, B., Ruszkowska-Ciastek, B., 2015. CRP, but not TNF-alpha or IL-6, decreases after weight loss in patients with morbid obesity exposed to intensive weight reduction and balneological treatment. Journal of Zhejiang University-Science B 16, 404-411.

Rosen, E.D., Hsu, C.-H., Wang, X., Sakai, S., Freeman, M.W., Gonzalez, F.J., Spiegelman, B.M., 2002. CEBPA induces adipogenesis through PPARG: a unified pathway. Genes Dev. 16, 22-26.

Rosen, E.D., MacDougald, O.A., 2006. Adipocyte differentiation from the inside out. Nature reviews Molecular cell biology 7, 885-896.

Rosen, E.D., Sarraf, P., Troy, A.E., Bradwin, G., Moore, K., Milstone, D.S., Spiegelman, B.M., Mortensen, R.M., 1999. PPARG is required for the differentiation of adipose tissue in vivo and in vitro. Mol. Cell 4, 611-617.

Rosen, E.D., Walkey, C.J., Puigserver, P., Spiegelman, B.M., 2000. Transcriptional regulation of adipogenesis. Genes Dev. 14, 1293-1307.

Ross, A., 1993. Symposium-retinoids-cellular-metabolism and activation-introduction, amer inst nutrition 9650 rockville pike, bethesda, md 20814.

Ross, A.C., Ternus, M.E., 1993. Vitamin A as a hormone: recent advances in understanding the actions of retinol, retinoic acid, and beta carotene. J. Am. Diet. Assoc. 93, 1285-1290.

Ross, A.C., Zolfaghari, R., 2004. Regulation of hepatic retinol metabolism: perspectives from studies on vitamin A status. The Journal of nutrition 134, 269S-275S.

Ross, S.E., Hemati, N., Longo, K.A., Bennett, C.N., Lucas, P.C., Erickson, R.L., MacDougald, O.A., 2000. Inhibition of adipogenesis by Wnt signaling. science 289, 950-953.

Ruiz, J., Antequera, T., Andres, A., Petron, M., Muriel, E., 2004. Improvement of a solid phase extraction method for analysis of lipid fractions in muscle foods. Anal. Chim. Acta 520, 201-205.

Ruiz, J., Cava, R., Antequera, T., Martín, L., Ventanas, J., López-Bote, C.J., 1998. Prediction of the feeding background of Iberian pigs using the fatty acid profile of subcutaneous, muscle and hepatic fat. Meat science 49, 155-163.

Safonova, I., Darimont, C., Amri, E.Z., Grimaldi, P., Ailhaud, G., Reichert, U., Shroot, B., 1994. Retinoids are positive effectors of adipose cell differentiation. Mol. Cell. Endocrinol. 104, 201-211.

Sagazio, A., Piantedosi, R., Alba, M., Blaner, W.S., Salvatori, R., 2007. Vitamin A deficiency does not influence longitudinal growth in mice. Nutrition 23, 483-488.

Saggerson, E.D., 1982. Carnitine acyltransferase activities in rat liver and heart measured with palmitoyl-CoA and octanoyl-CoA. Latency, effects of K+, bivalent metal ions and malonyl-CoA. Biochem. J 202, 397-405.

Sakaue, H., Konishi, M., Ogawa, W., Asaki, T., Mori, T., Yamasaki, M., Takata, M., Ueno, H., Kato, S., Kasuga, M., 2002. Requirement of fibroblast growth factor 10 in development of white adipose tissue. Genes Dev. 16, 908-912.

Samborski, A., Graf, A., Krebs, S., Kessler, B., Reichenbach, M., Reichenbach, H.-D., Ulbrich, S.E., Bauersachs, S., 2013. Transcriptome Changes in the Porcine Endometrium During the Pre-attachment Phase. Biol. Reprod., biolreprod. 113.112177.

Samuel, W., Kutty, R.K., Nagineni, S., Gordon, J.S., Prouty, S.M., Chandraratna, R.A., Wiggert, B., 2001. Regulation of stearoyl coenzyme A desaturase expression in human retinal pigment epithelial cells by retinoic acid. J. Biol. Chem. 276, 28744-28750.

Sanz, M., Lopez-Bote, C.J., Menoyo, D., Bautista, J.M., 2000. Abdominal fat deposition and fatty acid synthesis are lower and beta-oxidation is higher in broiler chickens fed diets containing unsatuarated rather than saturated fat. J. Nutr. 130, 3034-3037.

Sanz-Santos, G., Jiménez-Marín, Á., Bautista, R., Fernández, N., Claros, G.M., Garrido, J.J., 2011. Gene expression pattern in swine neutrophils after lipopolysaccharide exposure: a time course comparison, BMC proceedings, BioMed Central Ltd, p. S11.

Sara, V.R., Hall, K., 1990. Insulin-like growth factors and their binding proteins. Physiol. Rev. 70, 591-614.

Sargis, R.M., Johnson, D.N., Choudhury, R.A., Brady, M.J., 2010. Environmental endocrine disruptors promote adipogenesis in the 3t3- l1 cell line through glucocorticoid receptor activation. Obesity 18, 1283-1288.

Sato, M., Hiragun, A., 1988. Demonstration of 1α, 25- dihydroxyvitamin D3 receptor- like molecule in ST 13 and 3T3 L1 preadipocytes and its inhibitory effects on preadipocyte differentiation. Journal of cellular physiology 135, 545-550.

Schmidt, E.B., Dyerberg, J., 1994. Omega-3 fatty acids. Current status in cardiovascular medicine. Drugs 47, 405-424.

Schöne, F., 1986. Der Vitamin-A-Haushalt und die ihn beeinflussenden Faktoren unter besonderer Berücksichtigung des wachsenden Schweines, Mh. Vet. Med 41, 401-405.

Schwarz, E.J., Reginato, M.J., Shao, D., Krakow, S.L., Lazar, M.A., 1997. Retinoic acid blocks adipogenesis by inhibiting C/EBPbeta-mediated transcription. Mol. Cell. Biol. 17, 1552-1561.

Schweigert, F.J., Buchholz, I., Schuhmacher, A., Gropp, J., 2001. Effect of dietary β-carotene on the accumulation of β-carotene and vitamin A in plasma and tissues of gilts. Reprod. Nutr. Dev. 41, 47-55.

Scott, M., Nesheim, M., Young, R., 1982. Vitamins, In: Scott, M., Nesheim, M., Young, R. (Eds.), Nutrition of the chicken, 3rd edition, Scott and associates, Ithaca, New York, USA, pp. 119-147.

Seerley, R., Briscoe, J., McCampbell, H., 1978. A comparison of poultry and animal fat on performance, body composition and tissue lipids of swine. J. Anim. Sci. 46, 1018-1023.

Segura, J., Lopez-Bote, C.J., 2014. A laboratory efficient method for intramuscular fat analysis. Food Chem. 145, 821-825.

Sepe, A., Tchkonia, T., Thomou, T., Zamboni, M., Kirkland, J.L., 2011. Aging and regional differences in fat cell progenitors–a mini-review. Gerontology 57, 66-75.

Serra, X., Gil, F., Pérez-Enciso, M., Oliver, M., Vázquez, J., Gispert, M., Dıaz, I., Moreno, F., Latorre, R., Noguera, J., 1998. A comparison of carcass, meat quality and histochemical characteristics of Iberian (Guadyerbas line) and Landrace pigs. Livestock Production Science 56, 215-223.

Serrano, M., Valencia, D., Fuentetaja, A., Lázaro, R., Mateos, G., 2009. Influence of feed restriction and sex on growth performance and carcass and meat quality of Iberian pigs reared indoors. J. Anim. Sci. 87, 1676-1685.

Serrano, M., Valencia, D., Nieto, M., Lázaro, R., Mateos, G., 2008. Influence of sex and terminal sire line on performance and carcass and meat quality of Iberian pigs reared under intensive production systems. Meat science 78, 420-428.

Sethi, J., Vidal-Puig, A., 2010. Wnt signalling and the control of cellular metabolism. Biochem. J 427, 1-17.

Shahidi, F., Wanasundara, P., Hong, C., 1992. Antioxidant Activity of Phenolic-Compounds in Meat Model Systems, ACS symposium series, AMER CHEMICAL SOC 1155 16TH ST, NW, WASHINGTON, DC 20036, pp. 214-222.

Sharma, N., Gandemer, G., Goutefongea, R., 1987. Comparative lipid composition of porcine muscles at different anatomical locations. Meat science 19, 121-128.

Shi, X., Wallis, A.M., Gerard, R.D., Voelker, K.A., Grange, R.W., DePinho, R.A., Garry, M.G., Garry, D.J., 2012. Foxk1 promotes cell proliferation and represses myogenic differentiation by regulating Foxo4 and Mef2. J. Cell Sci. 125, 5329-5337.

Shimano, H., Shimomura, I., Hammer, R.E., Herz, J., Goldstein, J.L., Brown, M.S., Horton, J.D., 1997. Elevated levels of SREBP-2 and cholesterol synthesis in livers of mice homozygous for a targeted disruption of the SREBP-1 gene. J. Clin. Invest. 100, 2115.

Shimba, S., Ogawa, T., Hitosugi, S., Ichihashi, Y., Nakadaira, Y., Kobayashi, M., Tezuka, M., Kosuge, Y., Ishige, K., Ito, Y., 2011. Deficient of a clock gene, brain and muscle Arnt-like protein-1 (BMAL1), induces dyslipidemia and ectopic fat formation. PloS one 6, e25231.

Shimomura, Y., Tamura, T., Suzuki, M., 1990. Less body fat accumulation in rats fed a safflower oil diet than in rats fed a beef tallow diet. The Journal of nutrition 120, 1291-1296.

Shin, D.-J., McGrane, M.M., 1997. Vitamin A regulates genes involved in hepatic gluconeogenesis in mice: phosphoenolpyruvate carboxykinase, fructose-1, 6-bisphosphatase and 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase. The Journal of nutrition 127, 1274-1278.

Siddle, K., 2011. Signalling by insulin and IGF receptors: supporting acts and new players. Journal of molecular endocrinology 47, R1-R10.

Siebert, B., Kruk, Z., Davis, J., Pitchford, W., Harper, G., Bottema, C., 2006. Effect of low vitamin A status on fat deposition and fatty acid desaturation in beef cattle. Lipids 41, 365-370.

Siebert, B.D., Pitchford, W.S., Kruk, Z.A., Kuchel, H., Deland, M.P.B., Bottema, C.D.K., 2003. Differences in Delta (9) desaturase activity between jersey- and limousin-sired cattle. Lipids 38, 539-543.

Simopoulos, A.P., 2004. Omega-6/omega-3 essential fatty acid ratio and chronic diseases. Food Rev. Int. 20, 77-90.

Simopoulos, A.P., 2008. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp. Biol. Med. 233, 674-688.

Singh, I., Lazo, O., Dhaunsi, G., Contreras, M., 1992. Transport of fatty acids into human and rat peroxisomes. Differential transport of palmitic and lignoceric acids and its implication to X-adrenoleukodystrophy. J. Biol. Chem. 267, 13306-13313.

Skiba, G., Raj, S., Polawska, E., 2013. Profile of fatty acids and activity of elongase and Delta 5 and Delta 9 desaturase of growing pigs differ in concentration of intramuscular fat in musculus longissimus dorsi. Anim. Sci. Pap. Rep. 31, 123-137.

Sklan, D., Donoghue, S., 1982. Vitamin E response to high dietary vitamin A in the chick. The Journal of nutrition 112, 759-765.

Smas, C., Sul, H., 1996. Characterization of Pref-1 and its inhibitory role in adipocyte differentiation. International journal of obesity and related metabolic disorders: journal of the International Association for the Study of Obesity 20, S65-72.

Smith, S.B., Mersmann, H.J., Smith, E.O., Britain, K.G., 1999. Stearoyl-coenzyme A desaturase gene expression during growth in adipose tissue from obese and crossbred pigs. J. Anim. Sci. 77, 1710-1716.

Sobol, M., Krawczyńska, A., Skiba, G., Raj, S., Weremko, D., Herman, A., 2015. The effect of breed and feeding level on carcass composition, fatty acid profile and expression of genes encoding enzymes involved in fat metabolism in two muscles of pigs fed a diet enriched in n-3 fatty acids. A preliminary study. Journal of Animal and Feed Sciences 648, 127.

Sodhi, S.S., Song, K.-D., Ghosh, M., Sharma, N., Lee, S.J., Kim, J.H., Kim, N., Mongre, R.K., Adhikari, P., Kim, J.Y., 2014. Comparative transcriptomic analysis by RNA-seq to discern differential expression of genes in liver and muscle tissues of adult Berkshire and Jeju Native Pig. Gene 546, 233-242.

Soupene, E., Kuypers, F.A., 2008. Mammalian long-chain acyl-CoA synthetases. Exp. Biol. Med. 233, 507-521.

Spiegelman, B., Choy, L., Hotamisligil, G., Graves, R., Tontonoz, P., 1993. Regulation of adipocyte gene expresión in differentiation and sindromes of obesity/diabetes. J Biol Chem. 268.

St John, L., Young, C., Knabe, D., Thompson, L., Schelling, G., Grundy, S., Smith, S., 1987. Fatty acid profiles and sensory and carcass traits of tissues from steers and swine fed an elevated monounsaturated fat diet. J. Anim. Sci. 64, 1441-1447.

Steibel, J.P., Poletto, R., Coussens, P.M., Rosa, G.J.M., 2009. A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. Genomics 94, 146-152.

Stephensen, C.B., Blount, S.R., Schoeb, T.R., Park, J.Y., 1993. Vitamin-a-deficiency impairs some aspects of the host response to influenza-a virus-infection in balb/c mice. J. Nutr. 123, 823-833.

Stevenson, E.J., Giresi, P.G., Koncarevic, A., Kandarian, S.C., 2003. Global analysis of gene expression patterns during disuse atrophy in rat skeletal muscle. The Journal of physiology 551, 33-48.

Stöger, R., 2008. The thrifty epigenotype: an acquired and heritable predisposition for obesity and diabetes? Bioessays 30, 156-166.

Suarez-Belloch, J., Sanz, M., Joy, M., Latorre, M., 2013. Impact of increasing dietary energy level during the finishing period on growth performance, pork quality and fatty acid profile in heavy pigs. Meat Science 93, 796-801.

Sukhija, P.S., Palmquist, D.L., 1988. Rapid Method for Determination of Total Fatty-Acid Content and Composition of Feedstuffs and Feces. J. Agric. Food Chem. 36, 1202-1206.

Surles, R.L., Mills, J.P., Valentine, A.R., Tanumihardjo, S.A., 2007. One-time graded doses of vitamin A to weanling piglets enhance hepatic retinol but do not always prevent vitamin A deficiency. The American journal of clinical nutrition 86, 1045-1053.

Suryawan, A., Hu, C., 1997. Effect of retinoic acid on differentiation of cultured pig preadipocytes. J. Anim. Sci. 75, 112-117.

Sutherland, M., Rodriguez-Zas, S., Ellis, M., Salak-Johnson, J., 2005. Breed and age affect baseline immune traits, cortisol, and performance in growing pigs. J. Anim. Sci. 83, 2087-2095.

Suzuki, K., Shibata, T., Kadowaki, H., Abe, H., Toyoshima, T., 2003. Meat quality comparison of Berkshire, Duroc and crossbred pigs sired by Berkshire and Duroc. Meat science 64, 35-42.

Szabo, A.J., Grimaldi, R.D., de Lellis, R., 1973. Triglyceride synthesis by the human placenta. American Journal of Obstetrics & Gynecology 115, 263-266.

Tabor, H.K., Risch, N.J., Myers, R.M., 2002. Candidate-gene approaches for studying complex genetic traits: practical considerations. Nature Reviews Genetics 3, 391-397.

Takase, S., Ong, D.E., Chytil, F., 1986. Transfer of retinoic acid from its complex with cellular retinoic acid-binding protein to the nucleus. Arch Biochem Biophys 247, 328-334.

Talmant, A., Monin, G., Briand, M., Dadet, M., Briand, Y., 1986. Activities of metabolic and contractile enzymes in 18 bovine muscles. Meat Sci 18, 23-40.

Tan, B., Yin, Y., Liu, Z., Tang, W., Xu, H., Kong, X., Li, X., Yao, K., Gu, W., Smith, S.B., Wu, G., 2011. Dietary L-arginine supplementation differentially regulates expression of lipid-metabolic genes in porcine adipose tissue and skeletal muscle. J Nutr Biochem 22, 441-445.

Tanaka, T., Yoshida, N., Kishimoto, T., Akira, S., 1997. Defective adipocyte differentiation in mice lacking the CEBPB and/or CEBPD gene. The EMBO journal 16, 7432-7443.

Te Pas, M.F., Keuning, E., Van De Wiel, D.J., Young, J.F., Oksbjerg, N., Kruijt, L., 2011. Proteome profiles of Longissimus and Biceps femoris porcine muscles related to exercise and resting. Journal of Life Science 5, 598-608.

Tejeda, J., Gandemer, G., Antequera, T., Viau, M., Garcıa, C., 2002. Lipid traits of muscles as related to genotype and fattening diet in Iberian pigs: total intramuscular lipids and triacylglycerols. Meat Science 60, 357-363.

Tejerina, D., García-Torres, S., de Vaca, M.C., Vázquez, F., Cava, R., 2012. Effect of production system on physical–chemical, antioxidant and fatty acids composition of Longissimus dorsi and Serratus ventralis muscles from Iberian pig. Food Chem. 133, 293-299.

Thompson, M.R., Xu, D., Williams, B.R., 2009. ATF3 transcription factor and its emerging roles in immunity and cancer. Journal of molecular medicine 87, 1053-1060.

Tolson, K.P., Gemelli, T., Gautron, L., Elmquist, J.K., Zinn, A.R., Kublaoui, B.M., 2010. Postnatal Sim1 Deficiency Causes Hyperphagic Obesity and Reduced Mc4r and Oxytocin Expression. J. Neurosci. 30, 3803-3812.

Tolson, K.P., Gemelli, T., Meyer, D., Yazdani, U., Kozlitina, J., Zinn, A.R., 2014. Inducible neuronal inactivation of Sim1 in adult mice causes hyperphagic obesity. Endocrinology 155, 2436-2444.

Tontonoz, P., Hu, E., Spiegelman, B.M., 1994. Stimulation of adipogenesis in fibroblasts by PPARG2, a lipid-activated transcription factor. Cell 79, 1147-1156.

Torres-Rovira, L., Astiz, S., Caro, A., Lopez-Bote, C., Ovilo, C., Pallares, P., Perez-Solana, M., Sanchez-Sanchez, R., Gonzalez-Bulnes, A., 2012. Diet-induced swine model with obesity/leptin resistance for the study of metabolic syndrome and type 2 diabetes. The Scientific World Journal 2012.

Torres-Rovira, L., Pallares, P., Gonzalez-Añover, P., Perez-Solana, M.L., Gonzalez-Bulnes, A., 2011. The effects of age and reproductive status on blood parameters of carbohydrate and lipid metabolism in Iberian obese sows. Reproductive biology 11, 165-171.

Torres-Rovira, L., Tarrade, A., Astiz, S., Mourier, E., Perez-Solana, M., De La Cruz, P., Gomez-Fidalgo, E., Sanchez-Sanchez, R., Chavatte-Palmer, P., Gonzalez-Bulnes, A., 2013. Sex and breed-dependent organ development and metabolic responses in foetuses from lean and obese/leptin resistant swine. PloS one 8, 1-9.

Tous, N., Lizardo, R., Vila, B., Gispert, M., Font-i-Furnols, M., Esteve-Garcia, E., 2013. Effect of a high dose of CLA in finishing pig diets on fat deposition and fatty acid composition in intramuscular fat and other fat depots. Meat Sci 93, 517-524.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., Pachter, L., 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature protocols 7, 562-578.

Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., Van Baren, M.J., Salzberg, S.L., Wold, B.J., Pachter, L., 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature biotechnology 28, 511-515.

Tsutsumi, C., Okuno, M., Tannous, L., Piantedosi, R., Allan, M., Goodman, D., Blaner, W., 1992. Retinoids and retinoid-binding protein expression in rat adipocytes. J. Biol. Chem. 267, 1805-1810.

Tuohetahuntila, M., Spee, B., Kruitwagen, H.S., Wubbolts, R., Brouwers, J.F., van de Lest, C.H., Molenaar, M.R., Houweling, M., Helms, J.B., Vaandrager, A.B., 2015. Role of long-chain acyl-CoA synthetase 4 in formation of polyunsaturated lipid species in hepatic stellate cells. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 1851, 220-230.

Unruh, J., Friesen, K., Stuewe, S., Dunn, B., Nelssen, J., Goodband, R., Tokach, M., 1996. The influence of genotype, sex, and dietary lysine on pork subprimal cut yields and carcass quality of pigs fed to either 104 or 127 kilograms. J. Anim. Sci. 74, 1274-1283.

Uzun, A., Leslin, C.M., Abyzov, A., Ilyin, V., 2007. Structure SNP (StSNP): a web server for mapping and modeling nsSNPs on protein structures with linkage to metabolic pathways. Nucleic Acids Res. 35, W384-W392.

Van Laere, A.-S., Nguyen, M., Braunschweig, M., Nezer, C., Collette, C., Moreau, L., Archibald, A.L., Haley, C.S., Buys, N., Tally, M., 2003. A regulatory mutation in IGF2 causes a major QTL effect on muscle growth in the pig. Nature 425, 832-836.

Vander Heiden, M.G., Cantley, L.C., Thompson, C.B., 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. science 324, 1029-1033.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology 3, research0034.

Ventanas, J., Andrés, A.I., 2001. Tecnología del jamón Ibérico: de los sistemas tradicionales a la explotación racional del sabor y el aroma. Mundi-Prensa.

Ventanas, S., Ventanas, J., Jurado, A., Estevez, M., 2006. Quality traits in muscle biceps femoris and back-fat from purebred Iberian and reciprocal Iberian x Duroc crossbred pigs. Meat Science 73, 651-659.

Ventanas, S., Ventanas, J., Ruiz, J., 2007. Sensory characteristics of Iberian dry-cured loins: Influence of crossbreeding and rearing system. Meat science 75, 211-219.

Ventanas, S., Ventanas, J., Ruiz, J., Estévez, M., Pandalai, S., 2005. Iberian pigs for the development of high-quality cured products. Recent Research Developments in Agricultural & Food Chemistry, Vol. 6, 27-53.

Verardo, L., Nascimento, C., Silva, F., Gasparino, E., Martins, M., Toriyama, E., Faria, V., Botelho, M., Costa, K., Lopes, P., 2013. Identification and validation of differentially expressed genes from pig skeletal muscle. J. Anim. Breed. Genet. 130, 372-381.

SAS 9.2 version. 2010. In SAS user´s guide. SAS Institute Inc. Cary, NC.

Veum, V., Dankel, S., Gjerde, J., Nielsen, H., Solsvik, M., Haugen, C., Christensen, B., Hoang, T., Fadnes, D., Busch, C., 2012. The nuclear receptors NUR77, NURR1 and NOR1 in obesity and during fat loss. Int. J. Obesity 36, 1195-1202.

Vicente, J., Isabel, B., Cordero, G., Lopez-Bote, C., 2013. Fatty acid profile of the sow diet alters fat metabolism and fatty acid composition in weanling pigs. Anim. Feed Sci. Technol. 181, 45-53.

Vicente-Manzanares, M., Ma, X., Adelstein, R.S., Horwitz, A.R., 2009. Non-muscle myosin II takes centre stage in cell adhesion and migration. Nature reviews Molecular cell biology 10, 778-790.

Villafán-Bernal, J.R., Sánchez-Enríquez, S., Muñoz-Valle, J.F., 2011. Molecular modulation of osteocalcin and its relevance in diabetes (Review). Int. J. Mol. Med. 28, 283-293.

Villegas, R., Williams, S.M., Gao, Y.T., Long, J., Shi, J., Cai, H., Li, H., Chen, C.C., Tai, E.S., Hu, F., 2014. Genetic Variation in the Peroxisome Proliferator- Activated Receptor (PPAR) and Peroxisome Proliferator- Activated Receptor Gamma Co- activator 1 (PGC1) Gene Families and Type 2 Diabetes. Annals of human genetics 78, 23-32.

Vogel, S., Gamble, M., Blaner, W., 1999. Retinoid uptake, metabolism and transport, Springer Verlag: Heidelberg, Germany, pp. 31-96.

Voillet, V., SanCristobal, M., Lippi, Y., Martin, P.G.P., Iannuccelli, N., Lascor, C., Vignoles, F., Billon, Y., Canario, L., Liaubet, L., 2014. Muscle transcriptomic investigation of late fetal development identifies candidate genes for piglet maturity. BMC Genomics 15.

Vousden, K.H., Ryan, K.M., 2009. p53 and metabolism. Nature Reviews Cancer 9, 691-700.

Wabitsch, M., Hauner, H., Heinze, E., Teller, W.M., 1995. The role of growth hormone/insulin-like growth factors in adipocyte differentiation. Metabolism 44, 45-49.

Wadsack, C., Tabano, S., Maier, A., Hiden, U., Alvino, G., Cozzi, V., Hüttinger, M., Schneider, W.J., Lang, U., Cetin, I., 2007. Intrauterine growth restriction is associated with alterations in placental lipoprotein receptors and maternal lipoprotein composition. Am J Physiol-Endoc M 292, E476-E484.

Waldén, T.B., Hansen, I.R., Timmons, J.A., Cannon, B., Nedergaard, J., 2012. Recruited vs. nonrecruited molecular signatures of brown,"brite," and white adipose tissues. Am J Physiol-Endoc M 302, E19-E31.

Wallberg-Henriksson, H., Zierath, J.R., 2001. GLUT4: a key player regulating glucose homeostasis? Insights from transgenic and knockout mice. Mol. Membr. Biol. 18, 205-211.

Walters, E.M., Wolf, E., Whyte, J.J., Mao, J., Renner, S., Nagashima, H., Kobayashi, E., Zhao, J., Wells, K.D., Critser, J.K., 2012. Completion of the swine genome will simplify the production of swine as a large animal biomedical model. BMC medical genomics 5, 55.

Wang, G.G., Allis, C.D., Chi, P., 2007. Chromatin remodeling and cancer, Part I: Covalent histone modifications. Trends Mol. Med. 13, 363-372.

Wang, S., Zhou, G., Shu, G., Wang, L., Zhu, X., Gao, P., Xi, Q., Zhang, Y., Yuan, L., Jiang, Q., 2013. Glucose utilization, lipid metabolism and BMP-Smad signaling pathway of porcine intramuscular preadipocytes compared with subcutaneous preadipocytes. Cellular Physiology and Biochemistry 31, 981-996.

Wang, Y., Hudak, C., Sul, H.S., 2010. Role of preadipocyte factor 1 in adipocyte differentiation. Clinical lipidology 5, 109-115.

Wang, Y.-Z., Huang, Y.-N., Sun, K.-Y., Qi, J.-H., Xiang, L., 2011. Leptin gene transfer regulates fibromuscular development and lipid deposition in muscles via SIRT1, FOXO3a and PGC-1α in mice in vivo. Int. J. Mol. Med. 28, 617-623.

Wang, Z., Gerstein, M., Snyder, M., 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nature Reviews Genetics 10, 57-63.

Watanabe, H., Suzuki, H., Fukui, Y., 2010. Fertilizability, developmental competence, and chromosomal integrity of oocytes microinjected with pre-treated spermatozoa in mice. Reproduction 139, 513-521.

Weber, T., Richert, B., Belury, M., Gu, Y., Enright, K., Schinckel, A., 2006. Evaluation of the effects of dietary fat, conjugated linoleic acid, and ractopamine on growth performance, pork quality, and fatty acid profiles in genetically lean gilts. J. Anim. Sci. 84, 720-732.

West, K.P., LeClerq, S.C., Shrestha, S.R., Wu, L.S.-F., Pradhan, E.K., Khatry, S.K., Katz, J., Adhikari, R., Sommer, A., 1997. Effects of vitamin A on growth of vitamin A-deficient children: field studies in Nepal. The Journal of nutrition 127, 1957-1965.

Who, J., Consultation, F.E., 2003. Diet, nutrition and the prevention of chronic diseases. World Health Organ Tech Rep Ser 916.

Wickramasinghe, S., Cánovas, A., Rincón, G., Medrano, J.F., 2014. RNA-sequencing: a tool to explore new frontiers in animal genetics. Livest Sci 166, 206-216.

Wiegand, B., Sparks, J., Parrish, F., Zimmerman, D., 2002. Duration of feeding conjugated linoleic acid influences growth performance, carcass traits, and meat quality of finishing barrows. J. Anim. Sci. 80, 637-643.

Wijchers, P., Burbach, J., Smidt, M., 2006. In control of biology: of mice, men and Foxes. Biochem. J 397, 233-246.

Witte, D., Ellis, M., McKeith, F., Wilson, E., 2000. Effect of dietary lysine level and environmental temperature during the finishing phase on the intramuscular fat content of pork. J. Anim. Sci. 78, 1272-1276.

Wolf, G., 2001. Retinoic acid homeostasis: Retinoic acid regulates liver retinol esterification as well as its own catabolic oxidation in liver. Nutr. Rev. 59, 391-394.

Wolfe, R.G., Maxwell, C.V., Nelson, E.C., Johnson, R.R., 1977. Effect of dietary-fat level on growth and lipogenesis in colostrum deprived neonatal pig. J. Nutr. 107, 2100-2108.

Wolfe, W., Sanjur, D., 1988. Contemporary diet and body weight of Navajo women receiving food assistance: an ethnographic and nutritional investigation. J. Am. Diet. Assoc. 88, 822-827.

Wolpert, L., Tickle, C., Arias, A.M., 2015. Principles of development. Oxford university press.

Wood, J., Nute, G., Richardson, R., Whittington, F., Southwood, O., Plastow, G., Mansbridge, R., Da Costa, N., Chang, K., 2004. Effects of breed, diet and muscle on fat deposition and eating quality in pigs. Meat Science 67, 651-667.

Wood, J.D., Enser, M., Fisher, A.V., Nute, G.R., Sheard, P.R., Richardson, R.I., Hughes, S.I., Whittington, F.M., 2008. Fat deposition, fatty acid composition and meat quality: A review. Meat Science 78, 343-358.

Woollett, L.A., 2001. The origins and roles of cholesterol and fatty acids in the fetus. Current opinion in lipidology 12, 305-312.

Wu, X., Patki, A., Lara-Castro, C., Cui, X., Zhang, K., Walton, R.G., Osier, M.V., Gadbury, G.L., Allison, D.B., Martin, M., 2011. Genes and biochemical pathways in human skeletal muscle affecting resting energy expenditure and fuel partitioning. J. Appl. Physiol. 110, 746-755.

Wu, Z., Bucher, N., Farmer, S.R., 1996. Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta, and glucocorticoids. Mol. Cell. Biol. 16, 4128-4136.

Wu, Z., Xie, Y., Bucher, N., Farmer, S.R., 1995. Conditional ectopic expression of C/EBP beta in NIH-3T3 cells induces PPAR gamma and stimulates adipogenesis. Genes Dev. 9, 2350-2363.

Xia, S.-F., Duan, X.-M., Hao, L.-Y., Li, L.-T., Cheng, X.-R., Xie, Z.-X., Qiao, Y., Li, L.-R., Tang, X., Shi, Y.-H., 2015. Role of thyroid hormone homeostasis in obesity-prone and obesity-resistant mice fed a high-fat diet. Metabolism 64, 566-579.

Xing, J., Xing, F., Zhang, C., Zhang, Y., Wang, N., Li, Y., Yang, L., Jiang, C., Zhang, C., Wen, C., 2014. Genome-wide gene expression profiles in lung tissues of pig breeds differing in resistance to porcine reproductive and respiratory syndrome virus. PloS one 9.

Xing, K., Zhu, F., Zhai, L., Liu, H., Wang, Y., Wang, Z., Chen, S., Hou, Z., Wang, C., 2015. Integration of Transcriptome and Whole Genomic Resequencing Data to Identify Key Genes Affecting Swine Fat Deposition.

Yamamoto, M., Kuroiwa, A., 2003. Hoxa- 11 and Hoxa- 13 are involved in repression of MyoD during limb muscle development. Development, growth & differentiation 45, 485-498.

Yang, X., Koltes, J.E., Park, C.A., Chen, D., Reecy, J.M., 2015. Gene Co-Expression Network Analysis Provides Novel Insights into Myostatin Regulation at Three Different Mouse Developmental Timepoints. PloS one 10.

Yasuhara, K., Ohno, Y., Kojima, A., Uehara, K., Beppu, M., Sugiura, T., Fujimoto, M., Nakai, A., Ohira, Y., Yoshioka, T., 2011. Absence of heat shock transcription factor 1 retards the regrowth of atrophied soleus muscle in mice. J. Appl. Physiol. 111, 1142-1149.

Yeh, W.-C., Cao, Z., Classon, M., McKnight, S.L., 1995. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev. 9, 168-181.

Ying, H., Araki, O., Furuya, F., Kato, Y., Cheng, S.-Y., 2007. Impaired adipogenesis caused by a mutated thyroid hormone α1 receptor. Mol. Cell. Biol. 27, 2359-2371.

Yu, K., Mo, D., Wu, M., Chen, H., Chen, L., Li, M., Chen, Y., 2014. Activating transcription factor 4 regulates adipocyte differentiation via altering the coordinate expression of CCATT/enhancer binding protein β and peroxisome proliferator- activated receptor G. FEBS J. 281, 2399-2409.

Zhang, G.H., Lu, J.X., Chen, Y., Zhao, Y.Q., Guo, P.H., Yang, J.T., Zang, R.X., 2014. Comparison of the adipogenesis in intramuscular and subcutaneous adipocytes from Bamei and Landrace pigs. Biochem. Cell Biol. 92, 259-267.

Zhang, X., Zhang, G., Zhang, H., Karin, M., Bai, H., Cai, D., 2008. Hypothalamic IKKβ/NF-κB and ER stress link overnutrition to energy imbalance and obesity. Cell 135, 61-73.

Zhao, X., Mo, D., Li, A., Gong, W., Xiao, S., Zhang, Y., Qin, L., Niu, Y., Guo, Y., Liu, X., 2011. Comparative analyses by sequencing of transcriptomes during skeletal muscle development between pig breeds differing in muscle growth rate and fatness. PloS one 6, e19774.

Zhao, Y., Li, J., Liu, H., Xi, Y., Xue, M., Liu, W., Zhuang, Z., Lei, M., 2015. Dynamic transcriptome profiles of skeletal muscle tissue across 11 developmental stages for both Tongcheng and Yorkshire pigs. BMC Genomics 16, 377.

Zheng, Z., Zhang, C., Zhang, K., 2010. Role of unfolded protein response in lipogenesis. World journal of hepatology 2, 203.

Zhou, G., Wang, S., Wang, Z., Zhu, X., Shu, G., Liao, W., Yu, K., Gao, P., Xi, Q., Wang, X., 2010. Global comparison of gene expression profiles between intramuscular and subcutaneous adipocytes of neonatal landrace pig using microarray. Meat science 86, 440-450.

Zhou, X., Li, D., Yin, J., Ni, J., Dong, B., Zhang, J., Du, M., 2007. CLA differently regulates adipogenesis in stromal vascular cells from porcine subcutaneous adipose and skeletal muscle. J. Lipid Res. 48, 1701-1709.

Zolfaghari, R., Cifelli, C.J., Banta, M.D., Ross, A.C., 2001. Fatty acid Δ 5-desaturase mRNA is regulated by dietary vitamin A and exogenous retinoic acid in liver of adult rats. Arch Biochem Biophys 391, 8-15.

Zolfaghari, R., Ross, A.C., 2002. Lecithin : Retinol acyltransferase expression is regulated by dietary vitamin A and exogenous retinoic acid in the lung of adult rats. J. Nutr. 132, 1160-1164.

Zolfaghari, R., Ross, A.C., 2003. Recent advances in molecular cloning of fatty acid desaturase genes and the regulation of their expression by dietary vitamin A and retinoic acid. Prostaglandins, leukotrienes and essential fatty acids 68, 171-179.

Zuo, Z.Y., Luo, H.L., Liu, K., Jia, H.N., Zhang, Y.W., Jiao, L.J., Chang, Y.F., 2014. Dietary vitamin E affects alpha-TTP mRNA levels in different tissues of the Tan sheep. Gene 541, 1-7.

7.-ANEXO 1: MATERIAL SUPLEMENTARIO

Capítulo 1: Comparative analysis of muscle transcriptome between pig genotypes identifies genes and regulatory mechanisms associated to growth, fatness and metabolism.

S1 Table. Gene information, primer sequences, amplicon size and efficiency of genes selected for qPCR validation

S2 Table. Genes found differentially expressed between purebred (IB) and Duroc-crossbred (IBxDU) newborn pigs.

S3 Table. RNA-Seq and qPCR validation results and correlation coefficient (r) between the two used methodologies

S4 Table.Enriched biological functions identified by IPA software in purebred (IB) and Duroc-crossbred (IBxDU) Iberian pigs (p<0.01)

S5 Table. Canonical pathways enriched in IB and IBxDU animals based on DE genes and TFR identified either by IPA or by RIFs study

S6 Table: Variant analysis performed in selected regulators

Capítulo 2: Age, muscle and genetic type modify muscle transcriptome in pigs: effects on gene expression and regulatory factors involved in growth and metabolism.

S1- Differentially expressed genes conditional on age (birth vs four months).

S2- Enriched pathways in the set of DE genes conditional on age (birth vs four months of age).

S3- Transcription factors affecting gene expression of *Longissimus dorsi* muscle from newborn and four months old Iberian pigs.

S4- Differentially expressed genes conditional on genetic type (Iberian (IB) vs Duroc X Iberian (IBxDU)) at birth and at four months of age.

S5- Transcription factors affecting gene expression of *Longissimus dorsi* muscle from pure and Duroc-crossbred Iberian pigs at birth and four months of age.

S6. Enriched pathways in the set of differentially expressed genes and transcription factors conditional on genetic type at birth and four months of age.

S7- Differentially expressed genes conditional on muscle (*Longissimus dorsi* (LD) vs *Biceps femoris* (BF)).

S8- Enriched pathways in the set of differentially expressed genes conditional on muscle type (*Longissimus dorsi* (LD) vs *Biceps femoris* (BF)) at birth and four months of age.

8.-ANEXO 2:

OTRAS PUBLICACIONES RELACIONADAS CON ESTA TESIS

8.1- Gender-specific early postnatal catch-up growth after intrauterine growth retardation by food restriction in swine with obesity/leptin resistance. Gonzalez-Bulnes, A., Ovilo, C., Lopez-Bote, C.J., Astiz, S., Ayuso, M., Perez-Solana, M., Sanchez-Sanchez, R., Torres-Rovira, L., 2012. Reproduction 144, 269-278.

REPRODUCTION

RESEARCH

Gender-specific early postnatal catch-up growth after intrauterine growth retardation by food restriction in swine with obesity/leptin resistance

A Gonzalez-Bulnes, C Ovilo[1], C J Lopez-Bote[2], S Astiz, M Ayuso[2], M L Perez-Solana, R Sanchez-Sanchez and L Torres-Rovira

Departamento de Reproducción Animal, INIA, Avda Puerta de Hierro s/n, 28040 Madrid, Spain, [1]Departamento de Mejora Genetica Animal, INIA, Carretera de la Coruña, Km 7,5, 28040 Madrid, Spain and [2]Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

Correspondence should be addressed to A Gonzalez-Bulnes; Email: bulnes@inia.es

Abstract

The effects of undernutrition during pregnancy on prenatal and postnatal development of the offspring were evaluated in sows with obesity/leptin resistance. Females were fed, from day 35 of pregnancy onwards, a diet fulfilling either 100% (group control, $n=10$) or 50% of the nutritional requirements (group underfed, $n=10$). In the control group, maternal body weight increased during pregnancy ($P<0.05$) while it decreased or remained steady in the underfed group. At days 75 and 100 of gestation, plasma triglycerides were lower but urea levels were higher in restricted than in control sows ($P<0.05$ for both). Assessment of the offspring indicated that the trunk diameter was always smaller in the restricted group ($P<0.01$ at day 50, $P<0.005$ at days 75 and 100 and $P<0.0001$ at birth) while head measurements were similar through pregnancy, although smaller in the restricted than in the control group at birth ($P<0.05$). Newborns from restricted sows were also lighter than offspring from control females ($P<0.01$) and had higher incidence of growth retardation ($P<0.01$). Afterwards, during lactation, early postnatal growth in restricted piglets was modulated by gender. At weaning, males from restricted sows were still lighter than their control counterparts ($P<0.05$), while females from control and underfed sows were similar. Thus, the current study indicates a gender-related differential effect in the growth patterns of the piglets, with females from restricted sows evidencing catch-up growth to neutralise prenatal retardation and reaching similar development than control counterparts.

Reproduction (2012) **144** 269–278

Introduction

The pandemic increase in the prevalence of common obesity and metabolic alterations in human beings has been related to an interaction between genetic and environmental factors (Gonzalez-Bulnes *et al.* 2011). There is increasing evidence that environmental factors influencing obesity and associated diseases are especially critical during prenatal and early postnatal stages (Gonzalez-Bulnes & Ovilo 2012). Prenatal development, both in human and animal species, is dependant on an adequate placental supply of oxygen and nutrients (Wu *et al.* 2006, Vuguin 2007). Placental supply of nutrients is directly related to the nutritional status of the mother. Females with undernutrition will induce undernutrition of the conceptuses, causing deficiencies in their growth leading to intrauterine growth retardation (IUGR) and reduced birth weight. Individuals with IUGR, depending on the diet during the infantile and juvenile stages of life, will continue to be small at maturity or will become obese (Gonzalez-Bulnes & Ovilo 2012).

A large set of interventional studies, based on epidemiological evidences in human beings, have been developed in both laboratory and farm animals. Both rodents and swine are commonly used in obesity studies. Pig has the advantage of sharing several similarities with humans: omnivorous habits, propensity to sedentary behaviour and obesity, similar characteristics of metabolism and cardiovascular system and proportional organ sizes (Douglas 1972, Mahley *et al.* 1975, Lunney 2007, Spurlock & Gabler 2008). Among the different swine genotypes, there are ancient breeds that, conversely to modern lean pigs, have a huge tendency towards fat accumulation. Main examples of fatty pigs are Iberian and Mangalica breeds, which have developed a syndrome of leptin resistance similar to that described in human beings (Martin *et al.* 2008,

ISSN 1470–1626 (paper) 1741–7899 (online)

DOI: 10.1530/REP-12-0105
Online version via www.reproduction-online.org

8.2- Fetal and early-postnatal developmental patterns of obese-genotype piglets exposed to prenatal programming by maternal over-and undernutrition. Gonzalez-Bulnes, A., Ovilo, C., J Lopez-Bote, C., Astiz, S., Ayuso, M., L Perez-Solana, M., Sanchez-Sanchez, R., Torres-Rovira, L., 2013. Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders) 13, 240-249.

Send Orders for Reprints to reprints@benthamscience.net

240 *Endocrine, Metabolic & Immune Disorders - Drug Targets*, 2013, *13*, 240-249

Fetal and Early-Postnatal Developmental Patterns of Obese-Genotype Piglets Exposed to Prenatal Programming by Maternal Over- and Undernutrition

Antonio Gonzalez-Bulnes[1,*], Cristina Ovilo[2], Clemente J. Lopez-Bote[3], Susana Astiz[1], Miriam Ayuso[3], Maria L. Perez-Solana[1], Raul Sanchez-Sanchez[1] and Laura Torres-Rovira[1]

[1]Departamento de Reproducción Animal, INIA, Avda. Puerta de Hierro s/n. 28040 Madrid, Spain; [2]Departamento de Mejora Genetica Animal, INIA, Crta. de la Coruña, km 7,5. 28040 Madrid, Spain; [3]Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Ciudad Universitaria s/n. 28040 Madrid, Spain

Abstract: The present study evaluated the effect of nutritional imbalances during pregnancy, either by excess or deficiency, on fertility and conceptus development in obese-genotype swine (Iberian pig). Twenty-five multiparous sows were fed, from mating to farrowing, with a standard diet fulfilling either 1.6 folds their daily maintenance requirements for pregnancy (overfed group, n = 12) or only the 50% of such requirements (underfed group, n = 13). Ten out of 12 overfed but only two out of 13 underfed sows became pregnant ($P<0.005$). Fetal development was determined in the pregnant females at Days 35, 50, 75 and 90 of pregnancy. The embryos from undernourished sows were smaller than the embryos from overfed females as early as at 35 days of pregnancy ($P<0.05$) and remained smaller until Day 90 of gestation. However, at the end of pregnancy, there were significant changes in the developmental patterns of fetuses. Thus, weight and size of the offspring from both nutritional treatments were finally similar at delivery; the same was found at weaning. There was thereafter a sex-related effect on the growth during the early-postnatal period, with male piglets of both nutritional origins being significantly heavier and more corpulent at weaning that their sisters ($P<0.05$). In conclusion, fetal growth conditioned by malnutrition from periconceptional stages is mainly regulated at the end of the pregnancy, so that ensure an adequate body-weight and size and, therefore, the survival of the offspring. Afterwards, the early-postnatal development of the offspring is affected by sex, independently from nutritional origin, with male piglets growing faster than females.

Keywords: Developmental-programming, fertility, gender-effects, leptin-resistance, metabolism, obesity.

INTRODUCTION

There is increasing evidence supporting the hypothesis of the fetal origin of some adult diseases [1], the later called *Developmental Origin of Health and Disease* (DOHaD; [2]). The DOHaD hypothesis states that prenatal and early-postnatal conditions markedly determine juvenile growth, lifetime fitness/obesity and appearance of some non-transmissible diseases [3-5]. During prenatal development, the conceptus evolves throughout different dynamic stages from fertilization to implantation and later organogenesis and enlargement. In such stages, the conceptus is highly susceptible to changes in the availability of both oxygen and nutrients in its intrauterine environment. In a process named *prenatal programming*, the developing embryo is able to modify the expression of its genome, by developmental plasticity, for adapting to inadequate intrauterine conditions. These conditions are mainly related to maternal malnutrition, either by excess or deficiency. Hence, maternal malnutrition from very early stages of pregnancy, from the periconceptional period, has profound consequences on fetal and postnatal development and homeostasis.

However, the knowledge of alterations in developmental changes in phenotype during the pre- and post-natal periods is scarce and mostly based in epidemiological studies. Interventional studies are required, hence, for providing further information on concrete timing and mechanism of such developmental changes. Thus, research in animal models is indispensable. Models based in large animals, like sheep or pigs, have the advantages of adequate body size for imaging and sampling and suitable pregnancy length for serial screening of different embryo/fetal stages. Swine, like humans, has omnivorous habits and propensity to sedentary behavior and obesity; moreover, metabolism and gastrointestinal function of both species are similar [6-10]. An additional advantage of research in swine is its applicability to animal production and welfare.

In view of these considerations, the objective of the present study was the assessment of fetal and early postnatal phenotypes resulting after prenatal programming by either under- or overnutrition from the periconceptional period, by using a swine model, the Iberian pig, which is characterized by thrifty genotype, *leptin resistance* and propensity to obesity and associated alterations [11]. In the present trial,

*Address correspondence to this author at the Dpto. de Reproduccion Animal, INIA, Avda. Puerta de Hierro s/n. 28040-Madrid, Spain;
Tel: + 34 91 347 4022; Fax: + 34 91 347 4014; E-mail: bulnes@inia.es

1875-6786/13 $58.00+.00

8.3- Maternal malnutrition and offspring sex determine juvenile obesity and metabolic disorders in a swine model of leptin resistance. Barbero, A., Astiz, S., Lopez-Bote, C.J., Perez-Solana, M.L., Ayuso, M., Garcia-Real, I., Gonzalez-Bulnes, A., 2013.. PloS one 8, e78424.

OPEN ACCESS Freely available online

PLOS ONE

Maternal Malnutrition and Offspring Sex Determine Juvenile Obesity and Metabolic Disorders in a Swine Model of Leptin Resistance

Alicia Barbero[1], Susana Astiz[2], Clemente J. Lopez-Bote[1], Maria L. Perez-Solana[2], Miriam Ayuso[1], Isabel Garcia-Real[1], Antonio Gonzalez-Bulnes[2*]

1 UCM, Facultad de Veterinaria, Madrid, Spain, 2 INIA, Madrid, Spain

Abstract

The present study aimed to determine, in a swine model of leptin resistance, the effects of type and timing of maternal malnutrition on growth patterns, adiposity and metabolic features of the progeny when exposed to an obesogenic diet during their juvenile development and possible concomitant effects of the offspring sex. Thus, four groups were considered. A CONTROL group involved pigs born from sows fed with a diet fulfilling their daily maintenance requirements for pregnancy. The treated groups involved the progeny of females fed with the same diet but fulfilling either 160% or 50% of pregnancy requirements during the entire gestation (OVERFED and UNDERFED, respectively) or 100% of requirements until Day 35 of pregnancy and 50% of such amount from Day 36 onwards (LATE-UNDERFED). OVERFED and UNDERFED offspring were more prone to higher corpulence and fat deposition from early postnatal stages, during breast-feeding; adiposity increased significantly when exposed to obesogenic diets, especially in females. The effects of sex were even more remarkable in LATE-UNDERFED offspring, which had similar corpulence to CONTROL piglets; however, females showed a clear predisposition to obesity. Furthermore, the three groups of pigs with maternal malnutrition showed evidences of metabolic syndrome and, in the case of individuals born from OVERFED sows, even of insulin resistance and the prodrome of type-2 diabetes. These findings support the main role of early nutritional programming in the current rise of obesity and associated diseases in ethnics with leptin resistance.

Citation: Barbero A, Astiz S, Lopez-Bote CJ, Perez-Solana ML, Ayuso M, et al. (2013) Maternal Malnutrition and Offspring Sex Determine Juvenile Obesity and Metabolic Disorders in a Swine Model of Leptin Resistance. PLoS ONE 8(10): e78424. doi:10.1371/journal.pone.0078424

Editor: Michael Müller, Wageningen University, Netherlands

Received May 18, 2013; **Accepted** September 20, 2013; **Published** October 24, 2013

Funding: The experimental work was supported by funds from the Spanish Ministry of Economy and Competitiveness (project AGL2010-21991-C03-03), co-funded by FEDER. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: bulnes@inia.es

Introduction

Obesity and associated metabolic disorders are increasingly concerning issues. The World Health Organization (WHO) foresees that, by 2015, approximately 2.3 billion adults will be overweight and more than 700 million will be obese; the mortality rate due to diabetes will double between 2005 and 2030. The highest incidence of obesity and diabetes has been traditionally found in developed countries; currently, the incidence of these disorders is mainly increasing in rapidly developing regions, like China, India and countries of the Middle East. The situation is aggravated by a severe boost in the incidence of obesity at childhood in the last years. The number of overweight children under the age of five was over 42 million in 2010, according to WHO data; again, close to 35 million are living in developing countries.

Thus, there is an urgent necessity to tackle this health problem, by applying prevention strategies and focused treatments. Such actions need to be based on a thorough knowledge of obesity and its effects, by both observational and interventional research. However, interventional experimentation is not affordable in human beings and it needs to be performed on animal models. Most of the studies have been carried out in mice; however, the use of large animals (rabbit, sheep, pig) offers numerous profitable characteristics for translational studies. First, their body size allows application of the same imaging techniques routinely used in humans and serial sampling of large amounts of blood and tissues. Second, pathways regulating appetite, energy balance and adipogenesis are more similar to humans. Moreover, research in large animal species may provide valuable insights not only as a translational model for humans, but also directly for improving animal production, health and welfare. The most

8.4- Prenatal programming of obesity in a swine model of leptin resistance: modulatory effects of controlled postnatal nutrition and exercise. Barbero, A., Astiz, S., Ovilo, C., Lopez-Bote, C., Perez-Solana, M., Ayuso, M., Garcia-Real, I., Gonzalez-Bulnes, A., 2014. Journal of developmental origins of health and disease. 5, 248-258.

Journal of Developmental Origins of Health and Disease (2014), 5(3), 248–258.

doi:10.1017/S2040174414000208

ORIGINAL ARTICLE

Prenatal programming of obesity in a swine model of leptin resistance: modulatory effects of controlled postnatal nutrition and exercise

A. Barbero[1], S. Astiz[2], C. Ovilo[2], C. J. Lopez-Bote[1], M. L. Perez-Solana[2], M. Ayuso[1], I. Garcia-Real[1] and A. Gonzalez-Bulnes[2]*

[1]*Universidad Complutense de Madrid, Facultad de Veterinaria, Madrid, Spain*
[2]*Animal Reproduction Department, INIA, Madrid, Spain*

The main role of early nutritional programming in the current rise of obesity and associated diseases is well known. However, translational studies are mostly based in postnatal food excess and, thus, there is a paucity of information on the phenotype of individuals with prenatal deficiencies but adequate postnatal conditions. Thus, we assessed the effects of prenatal programming (comparing descendants from females fed with a diet fulfilling 100 or only 50% of their nutritional requirements for pregnancy) on gene expression, patterns of growth and fattening, metabolic status and puberty attainment of a swine model of obesity/leptin resistance with controlled postnatal nutrition and opportunity of exercise. Maternal restriction was related to changes in the relationships among gene expression of positive (insulin-like growth factors 1 and 2) and negative (myostatin) regulators of muscle growth, with negative correlations in gilts from restricted pregnancies and positive relationships in the control group. In spite of these differences, the patterns of growth and fattening and the metabolic features during juvenile growth were similar in control gilts and gilts from restricted pregnancies. Concomitantly, there was a lack of differences in the timing of puberty attainment. However, after reaching puberty and adulthood, females from restricted pregnancies were heavier and more corpulent than control gilts, though such increases in weight and size were not accompanied by increases in adiposity. In conclusion, in spite of changes in gene expression induced by developmental programming, the propensity for higher weight and adiposity of individuals exposed to prenatal malnutrition may be modulated by controlled food intake and opportunity of physical exercise during infant and juvenile development.

Received 22 October 2013; Revised 29 January 2014; Accepted 25 February 2014; First published online 26 March 2014

Key words: developmental programming, metabolism, obesity

Introduction

Obesity and associated disorders have been traditionally reported in people from developed countries. However, the most recent epidemiological studies indicate that their incidence is currently increasing at a high rate in individuals living in rapidly developing areas.[1] At the same time, although obesity is traditionally more prevalent in adults, the incidence of obesity and overweight is currently increasing at an alarming rate in childhood. The number of overweight children was over 42 million in 2010; around 35 million of these are living in developing countries (http://www.who.int/dietphysicalactivity/childhood/en/). These two facts indicate important changes in the prevalence, incidence and sociodemographic profile of the disease, pointing to childhood and youth from developing countries.

Young people from developing countries are characterized by having intrinsic ethnic features, by descending from ancestors adapted to food scarcity and by a current exposure to nutrients excess, mostly in the form of high caloric obesogenic diets. The consequences have been mainly studied in India, which is currently facing epidemics of obesity and diabetes.[2] Indian ethnicity is thought to have an adaptive *thrifty phenotype* for surviving in scarce food environment; a high percentage of Indian newborns are affected by intrauterine growth retardation (IUGR)[2–4] and the postnatal exposure of these children to diets abundant in amount and calories causes increased adiposity, insulin resistance (IR) and cardiometabolic risk as early as at eight to nine years of age.[4,5] The same increase in childhood obesity is being reported in other areas like Brazil,[6] China[7] and Middle East countries.[8,9]

These findings closely resemble data supporting the hypothesis of the Developmental Origin of Health and Disease (DOHaD),[10] which addresses that the interaction between genetic predisposition, nutrition of the conceptus during pregnancy and postnatal exposure to obesogenic environments (mainly inadequate nutrition and lack of physical activity) markedly determines juvenile growth, fitness/obesity and appearance of some metabolic diseases.

The study of potential interactions of DOHaD with the physiology and pathophysiology of a complex multi-factorial disease like obesity makes necessary the development of observational, mechanistic and interventional studies. Experimentation in humans is obviously limited by ethical issues and, therefore, research needs to be performed in animal models; specifically, for translational purposes, in mammalian species.[11] Most of the studies have been carried out in mice; however, the use of

*Address for correspondence: A. Gonzalez-Bulnes, Animal Reproduction Department, INIA, Avda. Puerta de Hierro s/n. 28040-Madrid, Spain. (Email bulnes@inia.es)

8.5- Early-postnatal changes in adiposity and lipids profile by transgenerational developmental programming in swine with obesity/leptin resistance. Gonzalez-Bulnes, A., Astiz, S., Ovilo, C., Lopez-Bote, C.J., Sanchez-Sanchez, R., Perez-Solana, M.L., Torres-Rovira, L., Ayuso, M., Gonzalez, J., 2014. J. Endocrinol. 223, M17-M29.

Thematic Research | A GONZALEZ-BULNES and others | Transgenerational programming in obese swine | 223:1 M17–M29

Early-postnatal changes in adiposity and lipids profile by transgenerational developmental programming in swine with obesity/leptin resistance

Antonio Gonzalez-Bulnes, Susana Astiz, Cristina Ovilo[1], Clemente J Lopez-Bote[2], Raul Sanchez-Sanchez, Maria L Perez-Solana, Laura Torres-Rovira[2], Miriam Ayuso[2] and Jorge Gonzalez[3]

Departamento de Reproducción Animal, INIA, Avenida Puerta de Hierro s/n, Madrid 28040, Spain
[1]Departamento de Mejora Genética Animal, INIA, Ctra. La Coruña km 7·5, Madrid 28040, Spain
[2]Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid 28040, Spain
[3]Micros Veterinaria, Campus de Vegazana, Leon 24007, Spain

Correspondence should be addressed to A Gonzalez-Bulnes
Email bulnes@inia.es

Journal of Endocrinology

Abstract

Maternal malnutrition during pregnancy, both deficiency and excess, induces changes in the intrauterine environment and the metabolic status of the offspring, playing a key role in the growth, status of fitness/obesity and appearance of metabolic disorders during postnatal life. There is increasing evidence that these effects may not be only limited to the first generation of descendants, the offspring directly exposed to metabolic challenges, but to subsequent generations. This study evaluated, in a swine model of obesity/leptin resistance, the existence and extent of transgenerational developmental programming effects. Pre- and postnatal development, adiposity and metabolic features were assessed in the second generation of piglets, descendant of sows exposed to either undernutrition or overnutrition during pregnancy. The results indicated that these piglets exhibited early-postnatal increases in adiposity and disturbances in lipid profiles compatible with the early prodrome of metabolic syndrome, with liver tissue also displaying evidence of paediatric liver disease. These features indicative of early-life metabolic disorders were more evident in the males that were descended from overfed grandmothers and during the transition from milk to solid feeding. Thus, this study provides evidence supporting transgenerational developmental programming and supports the necessity for the development of strategies for avoiding the current epidemics of childhood overweight and obesity.

Key Words
- animal-models
- developmental-programming
- leptin
- obesity

Journal of Endocrinology (2014) 223, M17–M29

Introduction

The incidence of obesity and associated cardiometabolic disorders, traditionally higher in developed countries, is also currently increasing in rapidly developing countries such as India, Brazil, China and Middle East countries (Scully 2012). It is proposed that the main cause is related to major and rapid lifestyle changes

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-14-0217

Published by Bioscientifica Ltd.

8.6- Feasibility of MRI and selection of adequate region of interest for longitudinal studies of growth and fatness in swine models of obesity. Barbero, A., Garcia-Real, I., Astiz, S., Ayuso, M., Lopez-Bote, C., Gonzalez-Bulnes, A., 2014. Diagnostic and interventional imaging. 95, 839-847.

Diagnostic and Interventional Imaging (2014) 95, 839—847

ORIGINAL ARTICLE / *Research and innovation*

Feasibility of MRI and selection of adequate region of interest for longitudinal studies of growth and fatness in swine models of obesity

A. Barbero[a], I. Garcia-Real[a], S. Astiz[b], M. Ayuso[c], C.J. Lopez-Bote[c], A. Gonzalez-Bulnes[b,*]

[a] *Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain*
[b] *Departamento de Reproducción Animal, INIA, Avda. Puerta de Hierro s/n, 28040 Madrid, Spain*
[c] *Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain*

KEYWORDS
Animal models;
Fatness;
Magnetic resonance imaging;
Obesity

Abstract
Purposes: To determine the feasibility of MRI for imaging subcutaneous and visceral adiposity in longitudinal studies in obese swine models (Iberian pig). To establish the anatomical regions of interest (ROIs) and measurement points (MPs) adequate for their evaluation through analyses on the inter-individual variability and over-time reproducibility and through the assessment of their reliability and validity by comparison with in vivo and ex vivo zoometric data.
Material and methods: Five male and five female pigs were used from four (live weight around 48 kg and back-fat depth around 18 mm) to eight months old (live weight 134 kg and back-fat depth around 48 mm). MRI was carried out with a Panorama 0.23T scanner (Philips Medical Systems, Best, Netherlands), using a body/spine XL coil.
Results: The ROIs of election for visualization of subcutaneous data are located from the cranial margin of left diaphragmatic crura to the lumbar vertebrae L3. Visceral adiposity may be equally evaluated from the vertebrae L1 to L3.
Conclusions: MRI allows the evaluation of subcutaneous and visceral fatness in a single acquisition, which improves animal welfare and time- and cost-efficiency and provides an accurate, consistent and repeatable procedure for sequential studies of adiposity in obese swine.

* Corresponding author. Tel.: +34 91 347 4022; fax: +34 91 347 4014.
E-mail address: bulnes@inia.es (A. Gonzalez-Bulnes).

http://dx.doi.org/10.1016/j.diii.2014.03.002

8.7- Longissimus dorsi transcriptome analysis of purebred and crossbred Iberian pigs differing in muscle characteristics. Óvilo, C., Benítez, R., Fernández, A., Núñez, Y., Ayuso, M., Fernández, A.I., Rodríguez, C., Isabel, B., Rey, A.I., López-Bote, C., 2014. BMC Genomics 15, 413.

Óvilo *et al. BMC Genomics* 2014, **15**:413
http://www.biomedcentral.com/1471-2164/15/413

BMC Genomics

RESEARCH ARTICLE Open Access

Longissimus dorsi transcriptome analysis of purebred and crossbred Iberian pigs differing in muscle characteristics

Cristina Óvilo[1*], Rita Benítez[1], Almudena Fernández[1], Yolanda Núñez[1], Miriam Ayuso[2], Ana Isabel Fernández[1], Carmen Rodríguez[1], Beatriz Isabel[2], Ana Isabel Rey[2], Clemente López-Bote[2] and Luis Silió[1]

Abstract

Background: The two main genetic types in Iberian pig production show important phenotypic differences in growth, fattening and tissue composition since early developmental stages. The objective of this work was the evaluation of muscle transcriptome profile in piglets of both genetic types, in order to identify genes, pathways and regulatory factors responsible for their phenotypic differences. Contemporary families coming from pure Iberian pigs (IB) or from crossing with Duroc boars (DU×IB) were generated. Piglets (14 from each genetic type) were slaughtered at weaning (28 days) and *longissimus dorsi* was sampled for composition and gene expression studies. RNA was obtained and hybridized to Affymetrix *Porcine Genechip* expression arrays.

Results: Loin muscle chemical composition showed significant differences between genetic types in intramuscular fat content (6.1% vs. 4.3% in IB and DU×IB animals, respectively, $P = 0.009$) and in saturated ($P = 0.019$) and monounsaturated fatty acid proportions ($P = 0.044$). The statistical analysis of gene expression data allowed the identification of 256 differentially expressed (DE) genes between genetic types (FDR < 0.10), 102 upregulated in IB and 154 upregulated in DU×IB. Transcript differences were validated for a subset of DE genes by qPCR. We observed alteration in biological functions related to extracellular matrix function and organization, cellular adhesion, muscle growth, lipid metabolism and proteolysis. Candidate genes with known effects on muscle growth were found among the DE genes upregulated in DU×IB. Genes related to lipid metabolism and proteolysis were found among those upregulated in IB. Regulatory factors (RF) potentially involved in the expression differences were identified by calculating the *regulatory impact factors*. Twenty-nine RF were found, some of them with known relationship with tissue development (*MSTN*, *SIX4*, *IRX3*), adipogenesis (*CEBPD*, *PPARGC1B*), or extracellular matrix processes (*MAX*, *MXI1*). Correlation among the expression of these RF and DE genes show relevant differences between genetic types.

Conclusion: These results provide valuable information about genetic mechanisms determining the phenotypic differences on growth and meat quality between the genetic types studied, mainly related to the development and function of the extracellular matrix and also to some metabolic processes as proteolysis and lipid metabolism. Transcription factors and regulatory mechanisms are proposed for these altered biological functions.

Keywords: Iberian pig, Transcriptome, Genetic type, Transcription factors, Growth, Meat quality, Metabolism

*Correspondence: ovilo@inia.es
[1]Dpto Mejora Genética Animal, INIA, Ctra Coruña km 7.5, Madrid 28040, Spain
Full list of author information is available at the end of the article

8.8- Prenatal programming in an obese swine model: sex-related effects of maternal energy restriction on morphology, metabolism and hypothalamic gene expression.

Ovilo, C., González-Bulnes, A., Benítez, R., Ayuso, M., Barbero, A., Pérez-Solana, M.L., Barragán, C., Astiz, S., Fernández, A., López-Bote, C., 2014. British Journal of Nutrition 111, 735-746.

British Journal of Nutrition (2014), **111**, 735–746
doi:10.1017/S0007114513002948

Prenatal programming in an obese swine model: sex-related effects of maternal energy restriction on morphology, metabolism and hypothalamic gene expression

Cristina Óvilo[1]*, Antonio González-Bulnes[2], Rita Benítez[1], Miriam Ayuso[3], Alicia Barbero[4], Maria L. Pérez-Solana[2], Carmen Barragán[1], Susana Astiz[2], Almudena Fernández[1] and Clemente López-Bote[3]

[1]*Departamento de Mejora Genética Animal, INIA, Ctra. La Coruña km 7·5, Madrid 28040, Spain*
[2]*Departamento de Reproducción Animal, INIA, Madrid, Spain*
[3]*Departamento de Producción Animal, Facultad de Veterinaria, UCM, Madrid, Spain*
[4]*Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, UCM, Madrid, Spain*

(Submitted 28 January 2013 – Final revision received 3 July 2013 – Accepted 3 August 2013 – First published online 5 September 2013)

Abstract

Maternal energy restriction during pregnancy predisposes to metabolic alterations in the offspring. The present study was designed to evaluate phenotypic and metabolic consequences following maternal undernutrition in an obese pig model and to define the potential role of hypothalamic gene expression in programming effects. Iberian sows were fed a control or a 50% restricted diet for the last two-thirds of gestation. Newborns were assessed for body and organ weights, hormonal and metabolic status, and hypothalamic expression of genes implicated in energy homeostasis, glucocorticoid function and methylation. Weight and adiposity were measured in adult littermates. Newborns of the restricted sows were lighter ($P < 0·01$), but brain growth was spared. The plasma concentration of TAG was lower in the restricted newborns than in the control newborns of both the sexes ($P < 0·01$), while the concentration of cortisol was higher in females born to the restricted sows ($P < 0·04$), reflecting a situation of metabolic stress by nutrient insufficiency. A lower hypothalamic expression of anorexigenic peptides (*LEPR* and *POMC*, $P < 0·01$ and $P < 0·04$, respectively) was observed in females born to the restricted sows, but no effect was observed in the males. The expression of *HSD11B1* gene was down-regulated in the restricted animals ($P < 0·05$), suggesting an adaptive mechanism for reducing the harmful effects of elevated concentrations of cortisol. At 4 and 7 months of age, the restricted females were heavier and fatter than the controls ($P < 0·01$). Maternal feed restriction induces asymmetrical growth retardation and metabolic alterations in the offspring. Differences in gene expression at birth and higher growth and adiposity in adulthood suggest a female-specific programming effect for a positive energy balance, possibly due to overexposure to endogenous stress-induced glucocorticoids.

Key words: **Prenatal programming: Energy balance: Fatness and obesity: Hypothalamic gene expression**

The obesity epidemic is becoming one of the most important public health problems in many parts of the world, as it is associated with an increased risk of multiple chronic diseases, including several of the major causes of death and disability in the developed world (diabetes, CVD, stroke, hypertension and certain cancers). A recent study has estimated a 30% increase in the prevalence of obesity and a 130% increase in the prevalence of severe obesity over the next two decades[1].

The aetiology of obesity is complex as it is a multifactorial condition in which genetic, environmental and interaction factors are involved[2–4]. Among the environmental signals, nutritional status is known to have a profound impact on the development of obesity, not only during the development of the obese phenotype but also at earlier stages. In fact, it has been hypothesised that a deficient nutritional environment during the critical period of perinatal development programmes whole-body energy homeostasis for optimal survival under nutritionally deficient conditions[5–7]. When prenatal adaptations are mismatched with the environment that the individual confronts later in life, they may lead to metabolic alterations[8]. This process is known as 'prenatal/fetal programming' or 'developmental origins of health and disease'[9]. Epigenetic processes are known to be involved in the mechanisms responsible for the programming of body weight (BW) homeostasis[10,11], in agreement with the thrifty epigenotype theory, which states that DNA sequence polymorphisms may play

Abbreviations: *BW*, body weight; *LEPR*, leptin receptor; *POMC*, pro-opiomelanocortin.

***Corresponding author:** C. Óvilo, fax +34 913478743, email ovilo@inia.es

British Journal of Nutrition